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Isolamento e caracterização de células tronco pulpares e o seu potencial na modulação do estresse oxidativo *in vitro*

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Mestre em Odontologia, área de concentração em Dentística.

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Dissertação apresentada, como requisito parcial, para obtenção do grau de Mestre em Odontologia, Programa de Pós-Graduação em Odontologia, Faculdade de Odontologia de Pelotas, Universidade Federal de Pelotas.

Data da defesa: nove de dezembro de dois mil e quinze, ás dez horas

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Dedico este trabalho ao meu avô por me inspirar a amar a ciência e ao meu trabalho, conforme ele o faz, no auge dos seus 90 anos

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“Só fizemos melhor aquilo que repetidamente insistimos em melhorar. A busca da excelência não deve ser um objetivo, e sim, um hábito.”

(ARISTÓTELES)

Notas Preliminares

A presente dissertação foi redigida segundo o Manual de Normas para Dissertações, Teses e Trabalhos Científicos da Universidade Federal de Pelotas de 2013, adotando o Nível de Descrição 4 – estrutura em Artigos, descrita no Apêndice D do referido manual. <http://sisbi.ufpel.edu.br/?p=documentos&i=7> Acesso em: 06 de novembro de 2015.

O projeto de pesquisa contido nesta dissertação é apresentado em sua forma final após qualificação realizada em 19 de setembro de 2014 e aprovado pela Banca Examinadora composta pelos Professores Doutores Fernanda Nedel, Sandra Beatriz Chaves Tarquínio, Marta Gazal e Rachel Krolow Santos Silva Bast (suplente).

Resumo

FERRÚA, Camila Perelló. **Isolamento e caracterização das SHEDs e DPSCs e o seu potencial na modulação do estresse oxidativo *in vitro*.** 2015. 173 páginas. Dissertação de Mestrado em Odontologia – Programa de Pós Graduação em Odontologia. Universidade Federal de Pelotas, Pelotas, 2015.

O isolamento de células tronco da polpa de dentes permanentes (DPSCs) necessita de técnicas padronizadas, a fim de obter uma uniformidade nas culturas celulares obtidas. Haja vista o potencial terapêutico dessas células na área da saúde, bem como a escassez de estudos que abordem a capacidade de modulação do estresse oxidativo via mecanismos intracelulares de células tronco, essa dissertação teve por objetivo identificar através de duas revisões sistemáticas, como o isolamento de DPSCs vem sendo conduzido; e como vem sendo realizada a manipulação dos dentes para o isolamento de DPSCs. Subsequentemente, foi proposta uma análise *in vitro*, em torno da capacidade das DPSCs e das células tronco da polpa de dentes decíduos exfoliados (SHEDs) em modular altos níveis de estresse oxidativo (EO). Os resultados obtidos apontam para uma inconsistencia dos dados no que tange as revisões sistemáticas. Ambas as revisões sugerem uma lacuna na literatura, evidenciada através da falta de padronização das técnicas de isolamento executadas e o procesamento do dente anterior ao isolamento das células tronco. Em detrimento aos resultados obtidos no ensaio *in vitro*, pode-se salientar que embora se saiba da necessidade de aprofundar os conhecimentos nessa área, as DPSCs e as SHEDs possuem capacidade de adaptação a um insulto com H_2O_2, podendo ser, futuramente, interessantes para aplicação em terapias celulares.

Palavras-chave: revisão sistemática; DPSCs; SHEDs; estresse oxidativo.

Abstract

FERRÚA, Camila Perelló. **Isolation and characterization of SHEDs and DPSCs and their potential in modulating oxidative stress *in vitro*.** 2015. 173 pages. Dissertação de Mestrado em Odontologia – Programa de Pós Graduação em Odontologia. Universidade Federal de Pelotas, Pelotas, 2015.

The isolation of dental pulp stem cells (DPSCs) needs standard techniques in order to obtain uniformity in cell cultures. Considering the therapeutic potential of these cells in health, as well as the scarcity of studies addressing oxidative stress modulation capacity, via intracellular mechanisms, of stem cells: this dissertation aimed to identify through two systematic reviews, how has DPSCs isolation been conducted; and how has teeth manipulation for DPSCs isoltation been performed. Subsequently, it was proposed an *in vitro* analysis, twords DPSCs and stem cells from human exfoliated deciduous teeth (SHEDs) capacity to modulat high levels of oxidative stress. Results point to an inconsistency of data regarding the systematic reviews. Both reviews suggest a gap in the literature, evidenced by the lack of standardization of the executed isolation techniques and tooth processing prior to stem cell isolation. In relation to the results obtained in the *in vitro* assay, it can be showed that although more knowledge is necessary in this area, DPSCs and SHEDs have the capacity to adapt to an insult with H_2O_2, which can be in the future interesting for cell therapy applications.

Key-words: systematic review; DPSCs: SHEDs; oxidative stress.

Sumário[1]

1 Introdução

Atualmente, as células tronco têm sido utilizadas nos estudos envolvendo terapia celular, engenharia tecidual e biologia molecular. Estas células têm diferentes aplicações que permitem a constante evolução na área da saúde. (D'AQUINO et al., 2008).

Tais propriedades tem possibilitado utilizar as mesmas na regeneração tecidual (LANGER e VACANTI et al., 1993), no reparo de cicatriz em calvária de ratos (SEO et al., 2008), nos processos inflamatórios (CHANG et al.,2005), no tratamento ortopédico (KHAN et al., 2012) e nas doenças autoimunes (MIGUEL DE et al., 2012).

Nesse contexto, células tronco adultas são consideradas uma abordagem viável visando a transição para a prática clínica (NEDEL et al., 2009). Atualmente, células tronco adultas têm sido obtidas do tecido pulpar de dentes decíduos (MIURA et al., 2003) e permanentes (GRONTHOS et al., 2000), devido ao fato de serem facilmente captadas via motivos ortodônticos, periodontais, cárie e exfoliação natural de decíduos. Assim, todos esses aspectos corroboram para o conhecimento de que, em curto prazo, a odontologia é a área da saúde com maior possibilidade de aplicações da engenharia tecidual (CHEN et al., 2012) e terapia celular (VALKO et al., 2007).

Com intuito de impedir o desequilíbrio entre a produção de espécies reativas de oxigênio e nitrogênio, em relação à capacidade de remoção dessas por parte do sistema antioxidante, coibindo a formação de um ambiente com altos níveis de Estresse Oxidativo (EO) (HALLIWELL et al., 2004; MONAGHAN et al., 2009), tornou-se imprescindível a busca por métodos capazes de neutralizar de forma eficaz o EO.

Dessa forma, uma nova alternativa proposta para combater o EO é o uso de células tronco mesenquimais (mesenchymal stem cells - MSCs). O transplante de MSCs tem se comprovado como uma estratégia terapêutica em indivíduos com doenças relacionadas a EO, como o infarto agudo do miocárdio, a isquemia cerebral e a diabetes (VALLE-PRIETO et al., 2010). A literatura tem mostrado que essas células possuem atividade antioxidante e adaptação a um ambiente de estresse muito melhor do que se comparadas a outros tipos

celulares (CAPLAN, et al. 2013; VALLE-PRIETO et al., 2010; ZHAO, et al. 2012).

No que tange as MSCs, como as células tronco de polpa dental, existe um aspecto decisivo para o desenvolvimento desses estudos, o qual consiste no fato de que essas células estão presentes em pequenas quantidades, o que torna indubitável a necessidade de seu crescimento *in vitro* para gerar quantidade suficiente a fim de proporcionar avanços práticos na área da saúde (CAPLAN et al., 2009). Desse modo, é imprescindível a necessidade de aprofundar o conhecimento a cerca das células tronco e do seu microambiente (nicho) no intuito de recriá-lo *in vitro*. Evidenciando a necessidade de promover métodos de isolamento adequados, a fim de na tentativa de mimetizar, *in vitro*, o nicho das células tronco.

2 Projeto de pesquisa

2.1 Antecedentes e Justificativa

Os Radicais Livres (RLs) são átomos ou compostos que contêm um ou mais elétrons desemparelhados em suas órbitas externas (FINKEL e HOLBROOK et al., 2000). Os RLs podem existir sob a forma de espécies reativas de nitrogênio (ERNs), de carbono, de cloro, de enxofre, de oxigênio (EROs), entre outras (VASCONCELOS et al., 2007). Contudo, as formas mais comuns de RLs são as EROs [superóxido (0_2^-)], peróxido de hidrogênio (H_20_2) e radical hidroxil (OH-) e as ERNs [óxido nítrico (-NO), peróxido nítrico (ONOO-), nitrato (NO_3-) e nitrito (NO_2-)] (FANG., 2004) (Figura 1).

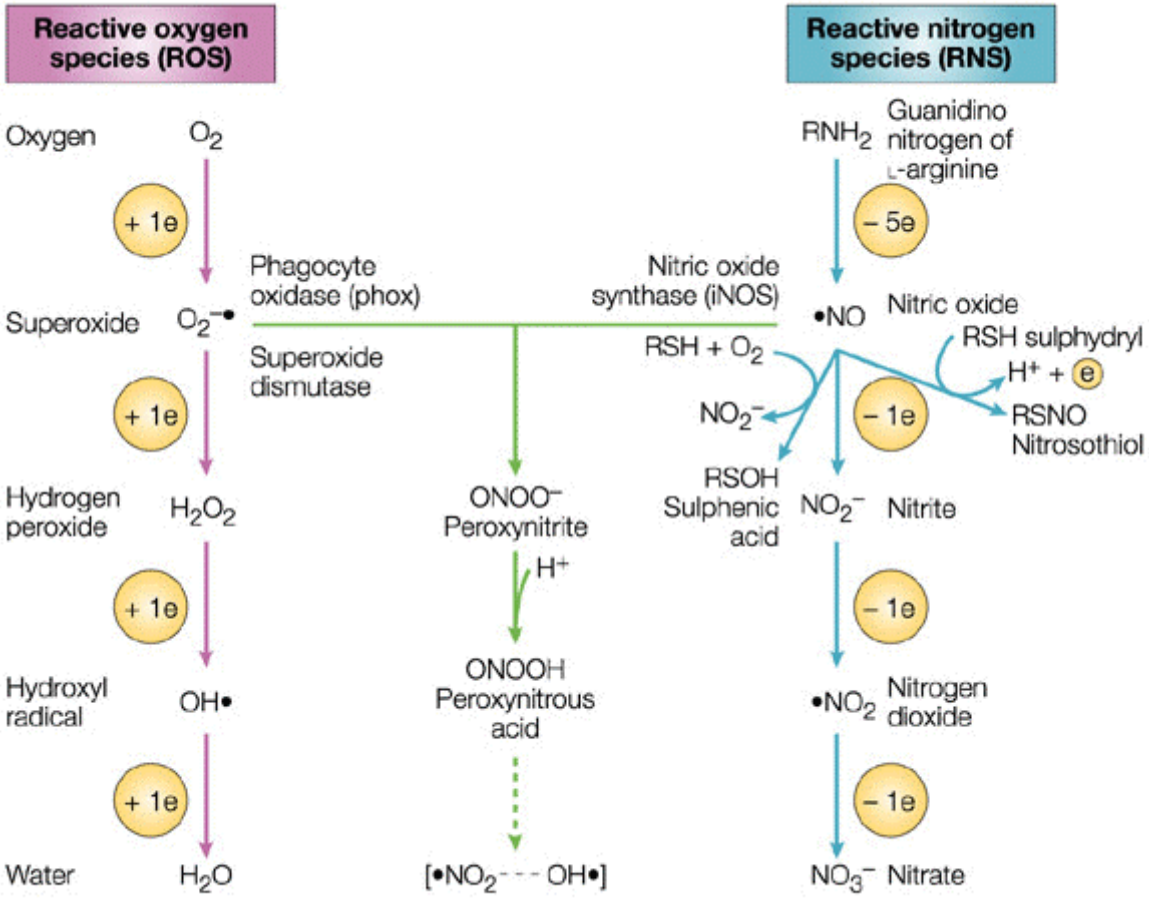

Figura 1: Formação de diferentes tipos de EROs e ERNs (Obtido de Fang, 2004).

A produção de RLs, EROs ou ERNs, acontece durante o funcionamento celular normal (VALKO et al., 2007), sobretudo, nas mitocôndrias, membranas

celulares e no citoplasma (BARBOSA et al., 2010). O processo que acontece a nível biomolecular é que durante a produção de energia celular, no interior da mitocôndria ocorre a redução de moléculas de oxigênio (O_2) em água (FANG et al., 2004).

A reação de fosforilação oxidativa ocorre no interior das mitocôndrias, com a finalidade de produzir adenosina trifosfato (ATP), reduzindo O_2 e consequentemente formando água. No entanto, durante a fosforilação oxidativa, muitos elétrons escapam e reduzem o O_2 ao O_2^-, principalmente nos sítios do complexo I e da coenzima Q (OHARA., 2006) (Figura 2).

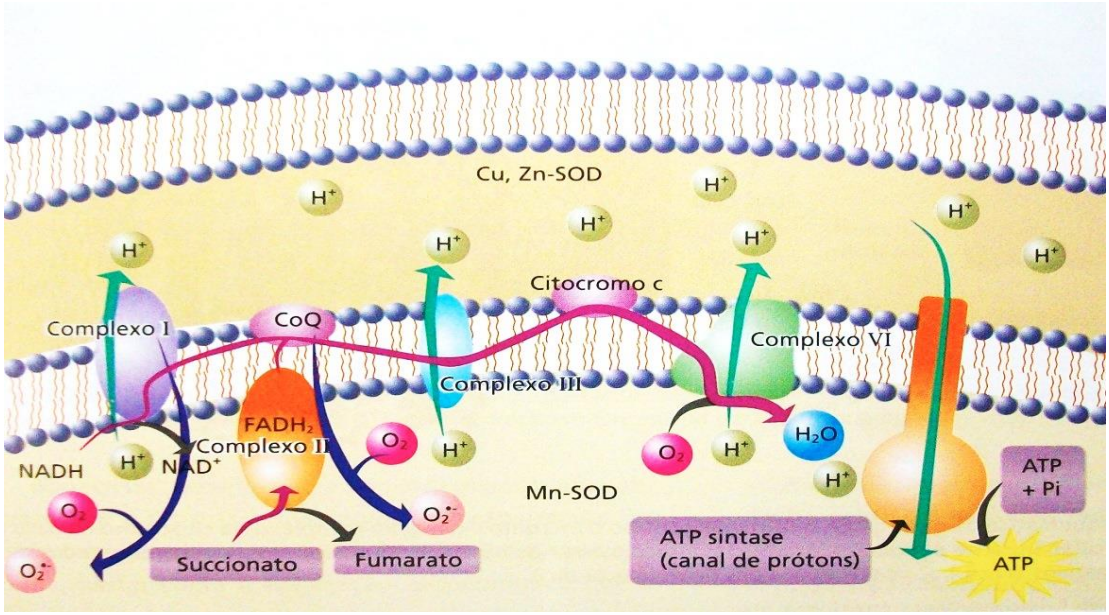

Figura 2: Cadeia transportadora de elétrons e produção de EROs, modelo esquemático da formação de EROs na mitocôndria (Obtido de OHARA., 2006).

A cadeia respiratória mitocondrial é composta por quatro complexos enzimáticos multipolipeptídicos e pode normalmente liberar em quantidades pequenas O_2^- e H_2O_2 através da auto-oxidação de uma ou mais espécies de flavina reduzida, complexo ferro-enxofre e ubiquinona gerados por succinato, dinucleótido-nicotinamida-adenina-reduzido (NADH) e outras ubiquinonas que reduzem desidrogenases. A cadeia respiratória é passível de sofrer com um ciclo vicioso que envolve lesões no DNA mitocondrial. Lesões do genoma mitocondrial podem causar mutações ou deleções dos produtos genéticos mitocondriais, levando a um aumento na formação de RLs. Essa situação proporciona um distúrbio no fluxo de elétrons na cadeia respiratória e provoca um escape de elétrons, resultando em um aumento ainda maior na geração de

RLs, tornando cada vez mais difícil o controle dessas espécies pelos mecanismos do corpo humano (NELSON e COX, 2010).

Embora a maioria das EROs pareça ser gerada através de produção endógena, fontes exógenas como a radiação ultravioleta, ozônio, poluentes (BALABAN et al., 2005), metais pesados, tabagismo e ingestão de álcool (BARBOSA et al., 2010) podem também ser responsáveis por desencadear o processo de formação de RLs (BALABAN et al., 2005; BARBOSA et al., 2010).

As EROs podem desempenhar no organismo um papel bastante relevante, atuando como mensageiros secundários, participando de vias de sinalização celular (HADDAD et al., 2002; HADDAD et al., 2002.2; RIBEIRO et al., 2005) e influenciando diretamente fatores de transcrição gênica (HADDAD et al., 2002; RIBEIRO et al., 2005). É importante salientar que esses mensageiros secundários representam cerca de 10% de todas EROs produzidas em células animais em condições normais, geralmente através de enzimas específicas como a nicotinamida-adenina-dinucleótido-fosfato (NADPH) (DRÖGE et al., 2002). As 90% restantes são geradas como um subproduto de processos metabólicos (BALABAN et al. 2005). No entanto dependendo de sua intensidade relativa as EROs são capazes de gerar danos aos tecidos (envelhecimento tecidual) (MONAGHAN et al., 2009; UCELLI et al., 2008), tendo sido relacionada com diversos tipos de patologias, como por exemplo osteoporose (SUN et al., 2013) e diabetes (CRUJEIRAS et al., 2013). Dessa forma, constata-se que a manutenção do equilíbrio entre a produção de RLs e as defesas antioxidantes é uma condição essencial para o comportamento normal do organismo (VALKO et al., 2007).

O desequilíbrio entre a produção de EROs ou ERNs em relação a capacidade de remoção dessas por parte do sistema antioxidante pode ser definido como Estresse Oxidativo (EO) (HALLIWELL et al., 2004; MONAGHAN et al., 2009). Portanto, a quantidade dessas espécies não é decisiva, uma vez que a relação entre a produção e a remoção de RLs aconteça de forma efetiva (MONAGHAN et al., 2009). Considerando-se que as mitocôndrias são as principais produtoras de EROs, e que também sofrem com os efeitos dessa capacidade destrutiva, observa-se a formação de um ciclo vicioso em que esta organela produz espécies reativas e são afetadas por elas, produzindo cada

vez mais material oxidativo (FINKEL e HOLBROOK, 2000; NELSON e COX., 2010). O descontrole do EO afeta significativamente moléculas biológicas essenciais como o DNA, as proteínas e os lipídios levando à sua modificação e frequentemente à inutilização, inibindo sua função normal (VALKO et al., 2007; RIDNOUR et al., 2005).

Os tipos de danos oxidativos causados pelas EROs à biomoléculas podem ser divididos em 3 categorias principais: lipoperoxidação, dano oxidativo ao ácido desoxirribonucléico (DNA) e oxidação de proteínas. A lipoperoxidação trata-se de um processo no qual ocorre a oxidação das cadeias de ácidos graxos polinssaturadas. Ocorre a abstração de átomos de hidrogênio de um ácido graxo poliinsaturado, que leva à formação de um dieno conjugado por rearranjo molecular. Esta molécula pode sofrer o ataque de O_2, formando um radical peroxil (ROO-). Este radical pode continuar o ciclo de lipoperoxidação através da abstração de átomos de hidrogênio de cadeias poliinsaturadas próximas, transformando-se em um peróxido lipídico (LOOH). Este reinicia o processo, gerando uma reação oxidativa em cadeia. Esses radicais são danosos, uma vez que podem fragmentar proteínas, formando grupos carbonilas. Na presença de calor ou de íons metálicos, os peróxidos aminoacídicos podem formar novos radicais orgânicos, gerando reações cíclicas. Além disso, podem atacar grupos tiólicos de outras proteínas, e sua degradação é considerada bastante difícil, por não serem substratos para enzimas antioxidantes, como a catalase (CAT), a superóxido desmutase (SOD) e a glutationa peroxidase (GPx) (NELSON e COX., 2010) (Figura 3).

As proteínas sofrem danos através de EROs e moléculas originadas de processos de oxidação que atacam diretamente a sua estrutura. Ligações peptídicas podem ser atacadas na abstração de hidrogênio pelo radical hidroxil (-OH). O -OH demanda uma atenção especial, haja vista que exerce efeitos nocivos às proteínas. Inicialmente, o ataque de -OH pode gerar outros radicais capazes de combinar com o O_2, formando radicais alcoxilas (LO-) e ROO-, os quais podem fazer a abstração de –H e formar peróxidos nas cadeias de proteínas (HALLIWELL & GUTTERIDGE, 2007).

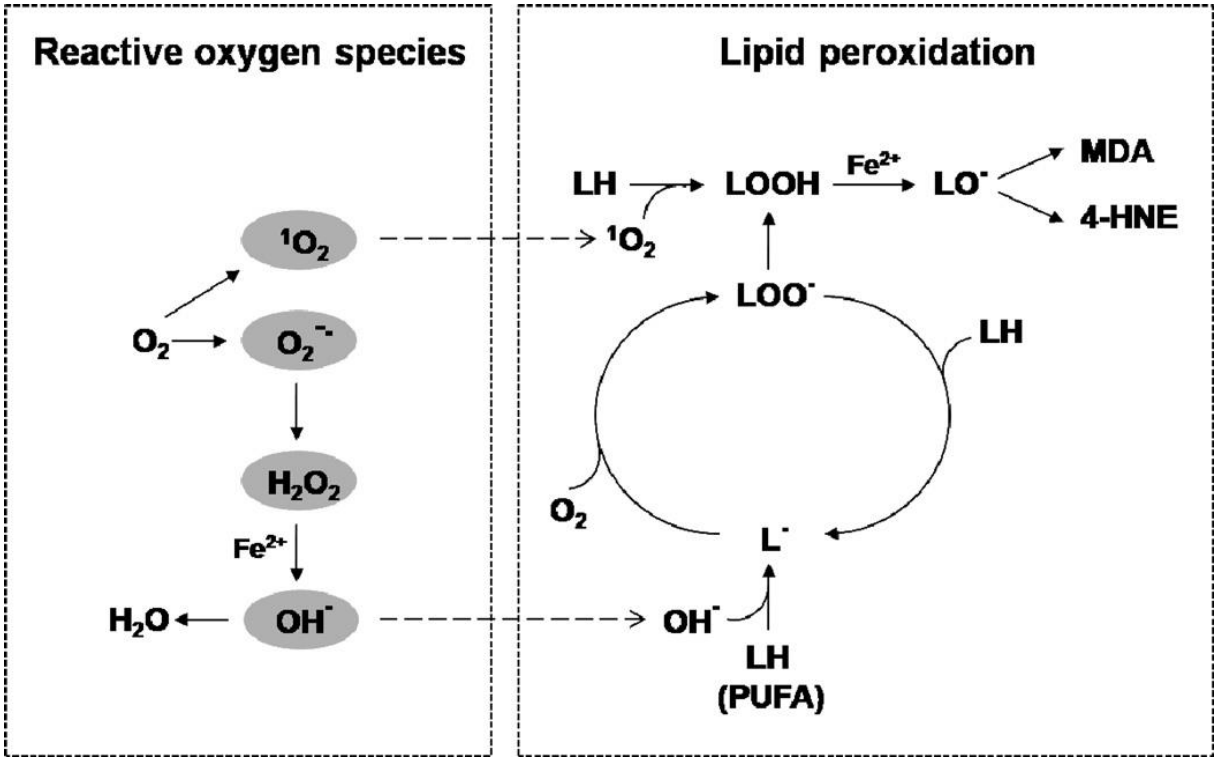

Figura 3: Formação de EROs e início e propagação da peroxidação lipídica (Obtido de CHEN et al., 2012).

Os RLs estão envolvidos em mutações, carcinogênse, envelhecimento e morte celular, através de alterações químicas e quebras de ligações nas bases nitrogenadas e na ribose do DNA. A ERO mais danosa ao DNA é o –OH, tendo em vista que é capaz de adicionar ligações duplas em bases heterocíclicas de DNA, além de abstrair hidrogênio da base nitrogenada timina e de cada um dos carbonos da desoxirribose. A abstração de átomos de hidrogênio feita pela -OH leva à formação de radicais de carbono, que na presença de oxigênio são convertidos a radicais peroxil de açúcares. Além disso, algumas proteínas nucleares podem ser atacadas pelo radical -OH, e realizar ligações cruzadas com o DNA, ocasionando falhas no reparo celular, replicação, transcrição e descondensamento da cromatina (DIZDAROGLU et al., 2002).

A concentração de RLs é controlada, por um sistema antioxidante, o qual é composto por duas vias, enzimática e não enzimática, ambas capazes de neutralizar as EROs (SHAMI et al., 2004; VALKO et al., 2007), impedir que possíveis danos oxidativos se amplifiquem e culminem em danos sistêmicos irreparáveis (SHAMI et al., 2004; BARBOSA et al., 2010). O sistema enzimático

encontra-se espalhado por todo o organismo, no meio intra e extracelular (VALKO et al., 2007), incluindo enzimas como a CAT, SOD e a GPx (VALKO et al., 2007; ZHOU et al., 2013).

A CAT é uma enzima intracelular responsável por decompor o H_2O_2, produto gerado pelo metabolismo celular quando exposto ao O_2 atmosférico, que em excesso é prejudicial, pois origina a EROs. Uma das fontes de H_2O_2 é a beta-oxidação de ácidos-graxos, necessária para a produção de alguns metabólitos essenciais. Dessa forma, a CAT transforma o H_2O_2 em uma substância inócua, o decompondo em água e O_2 (HALLIWEL et al., 2004), prevenindo a formação de bolhas de dióxido de carbono (CO_2) na corrente sanguínea (JIN et al., 2001).

Além da CAT, a GPx também é uma enzima com função de eliminação de H_2O_2. A GPx está relacionada à função antioxidante da glutationa na forma reduzida (GSH) com atividade peroxidásica. Para a manutenção do ambiente redutor intracelular a razão entre glutationa reduzida e oxidada (GSH/GSSG) deve ser mantida em níveis muito altos. Para evitar a depleção da GSH e aumento da GSSG, a glutationa redutase (GR) reduz a GSSG à custa de NADPH, regenerando a GSH e mantendo desta forma o estado redox intracelular (HALLIWEL et al., 2004).

Por fim, não menos relevante, a SOD, que tem por função proteger o citocromo c ao reduzir o O_2-, diminuindo a presença de RLs (NELSON e COX, 2013). O mecanismo de reação se inicia com a redução da SOD pelo O_2-, resultando na formação de O_2. A enzima reduzida reage novamente com outro O_2-, formando o H_2O_2 como produto, que subsequentemente sofre dismutação, dando origem a moléculas de água e O_2 (ALBERTS, et al., 2010; JOHANSEN et al., 2005; NELSON e COX, 2013).

Um evento que indica a atuação do sistema antioxidante enzimático no corpo humano é quando uma célula é desprovida de O_2, em estado de hipóxia, por exemplo, em um acidente vascular ou em um ataque cardíaco. Existe um desequilíbrio entre a chegada de elétrons a partir da oxidação de combustíveis celulares na matriz mitocondrial e a transferência de elétrons para o oxigênio molecular, levando a uma maior formação de EROs. No entanto, o organismo humano possui linhas de defesa enzimáticas contra as EROs. Em condições hipóxicas, o fator de hipóxia induzível (HIF-1) é sintetizado em grande

quantidade e age como um fator de transcrição, aumentando a síntese do transportador de glicose, de enzimas glicolíticas, da piruvato-desidrogenase (PDH), da protease que degrada a subunidade da COX4-1 e COX4-2 da citocromo-oxidase. Dessa forma, essas mudanças opõem-se à formação de EROs, por diminuírem o suprimento das formas reduzidas de NADH e de dinucleotídeo de flavina-adenina (FAD), além de efetivar a citocromo-oxidase. Sumariza-se assim que o HIF-1 é capaz de regular a expressão gênica para reduzir a formação de EROs (NELSON e COX, 2013).

O sistema não enzimático, por sua vez, é constituído por substâncias de origem endógena e da dieta. Alimentos de uma dieta que inclui especialmente, o consumo de frutas e vegetais, em que estejam presentes o ácido ascórbico (vitamina C), alfa-tocoferol (vitamina E), GPX, alguns flavonóides e carotenóides. Uma dieta a base de alimentos com propriedades antioxidantes pode estar associada à diminuição do risco de desenvolvimento de doenças relacionadas ao acúmulo de RLs e instalação de EO (VALKO et al., 2007; ZHOU et al., 2013).

Um recente estudo sugere que uma dieta suplementada com alfa-tocoferol, ácido ascórbico e selênio é capaz de otimizar a resposta antioxidante humana, melhorando os resultados, ainda que de forma modesta, dos testes de função hepática. Entretanto, observou-se que o combate aos RLs é bastante distinto entre os participantes da pesquisa. Indivíduos obesos, crianças e adolescentes não responderam satisfatoriamente a incorporação de suplementos na dieta e apesar das correlações significativas entre EO e inflamação, não houve redução nos marcadores de inflamação sistêmica (MURER et al., 2014). Pessoas obesas requerem um cuidado especial, uma vez que a inflamação promovida pela disfunção do tecido adiposo, induzida pela obesidade, tende a gerar um ambiente de EO, que tem sido associado ao desenvolvimento de tumores, como o de mama e diabetes mellitus, do tipo II (CRUJEIRAS, et al. 2013).

A fim de desenvolver novas alternativas de tratamento para algumas doenças que afetam e são comuns à população mundial, células tronco mesenquimais (MSCs) são comumente estudadas com intuito de desenvolver avanços em áreas como a terapia celular, engenharia tecidual e biologia molecular. As MSCs possuem fácil acessibilidade e disponibilidade, alta

plasticidade e capacidade de auto-renovação (D'AQUINO et al., 2009), e graças as suas características vantajosas podem ser associadas a resultados positivos em tratamentos ortopédicos (KHAN et al., 2012), doenças auto-imunes (DE MIGUEL et al., 2012) e processos inflamatórios (CHANG et al., 2005). Além disso, MSCs desempenham uma função moduladora e supressiva sobre a resposta imune do hospedeiro, apontando para a prevenção de rejeição em casos de transplantes (UCCELLI et al., 2008).

Embora seja consenso na literatura que as MSCs são bastante vantajosas, a sua utilização ainda deve ser analisada com cautela acerca da possibilidade da geração de danos como o desenvolvimento de tumores (DJOUAD et al., 2003). As MSCs ainda podem ser acometidas por aneuploidias, devido ao processo de senescência celular e redução dos telômeros, devido ao excesso de EROs (ESTRADA et al., 2013; RICHTER e VON ZGLINICKI 2007). Sabe-se que doenças como a osteoporose, podem estar relacionadas ao EO, de modo que resultem em alterações na capacidade proliferativa e de diferenciação celular e seja responsável pela apoptose de células regenerativas, como MSCs e osteócitos, levando à diminuição da densidade óssea (ALMEIDA et al., 2013).

É sabido que o corpo humano possui excelente maquinaria, sendo capaz de regular EROs produzidas de modo controlado. Entretanto, sabe-se que esse mecanismo de defesa natural nem sempre consegue desempenhar sua função de forma eficaz (VALKO et al., 2007). Uma vez que o EO pode causar danos celulares (envelhecimento) (MONAGHAN et al., 2009) e desencadear vários tipos de cânceres, diabetes, cirrose, doenças cardiovasculares e desordens do foro neurológico (FINKEL e HOLBROOK, 2000; VALKO et al., 2007), tornou-se imprescindível a busca por métodos capazes de neutralizar de forma eficaz o EO.

Dessa forma, uma nova alternativa proposta para combater o EO é o uso de MSCs. O transplante de MSCs tem se comprovado como uma estratégia terapêutica em indivíduos com doenças relacionadas a EO, como o infarto agudo do miocárdio, a isquemia cerebral e a diabetes (VALLE-PRIETO et al., 2010). A literatura tem mostrado que essas células possuem atividade antioxidante e adaptação a um ambiente de estresse muito mais elevadas se comparadas a outros tipos celulares (CAPLAN, et al. 2013; VALLE-PRIETO et

al., 2010; ZHAO, et al. 2012). Estas células apresentam altos níveis intracelulares de GSx (glutationa total), expressam nível elevado de metionina sulfóxido redutase A, uma enzima crucial para o reparo de proteínas oxidadas e para a recuperação de resíduos de metionina, que atuam como agentes eliminadores de oxidantes. Tem sido demonstrado que as MSCs expressam enzimas requeridas para o reparo do DNA. Assim as MSCs possuem a maquinaria enzimática e não-enzimática básica para detoxificar espécies reativas e para corrigir danos promovidos pelo EO do proteoma e genoma, garantindo o manejo eficiente do EO (VALLE-PRIETO et al., 2010).

Zhou e colaboradores em ensaio com modelo animal demonstrou que com as MSCs é possível melhorar a condição de saúde de ratos com lesão renal aguda induzida por cisplatina. Isso se mostrou possível, pela capacidade das MSCs em resistir ao EO, reduzindo a formação de alguns produtos nocivos, como 8-hidroxi-2'-desoxiguanosina (8-OHdG), malondialdeído (MDA) e GSH. Como resultado observou-se aumento na proliferação de células renais, redução na quantidade de apoptose dessas células e reparo dos tecidos comprometidos pela ocorrência da lesão renal aguda (ZHOU et al., 2013). Outro estudo afirma que a capacidade das MSCs em combater o EO *in vitro* e *in vivo*, pode ocorrer ainda por meio de atividade parácrina (LIU et al., 2012).

As células tronco mesenquimais oriundas de tecido adiposo (ADSCs) tem se mostrado aliadas daquelas pessoas preocupadas com aspectos estéticos, haja vista que essas células têm, através de fatores antioxidantes, reduzido à morte de fibroblastos, atuando como um mecanismo anti-envelhecimento da pele humana. Nesse estudo Kim e colaboradores, em 2008, constataram que as ADSCs possuem um efeito dérmico protetor, devido sua alta capacidade antioxidante, perceptível através da detecção de suas várias proteínas antioxidantes, bem como do aumento da atividade da SOD e da GPx (KIM et al., 2008).

As MSCs tem mostrado a capacidade de reduzir a morte por apoptose e o EO em animais propensos a hipertensão e a acidente vascular cerebral (AVC), em consequência do potencial neuroprotetor desempenhado pelas MSCs. Evidenciou-se o aumento da expressão do gene Bcl-2, anti-apoptótico, prevenindo a morte neural por apoptose, bem como a redução da peroxidação

lipídica em animais submetidos ao tratamento com MSCs, de apenas 30 dias. Esse tratamento, não muito complexo, consistiu em inserir MSCs diluídas em um tampão fosfato salino (PBS), na cisterna magna através da membrana atlanto occiptal em ratos com propensão espontânea a hipertensão (CALIÓ et al., 2014).

Uma pesquisa recente utilizando células tronco da polpa de dentes permanentes (DPSCs) investigou o efeito de um sistema de entrega de uma isoenzima da SOD (SOD 1), conjugada a um peptídeo de penetração celular de baixo peso molecular. O objetivo desse conjugado foi reduzir a senescência celular induzida por H_2O_2 e o EO após penetrar nas DPSCs. E como resultados, esse estudo sugere que o conjugado desenvolvido é eficaz para atenuar a senescência celular e tem capacidade de reverter a diferenciação de osteoblastos oriundos de DPSCs, através da inibição do EO (CHOI et al., 2012). Assim, pode-se propor que exista um potencial de estudos a ser melhor desenvolvido associando as DPSCs e as células tronco da polpa de dentes decíduos exfoliados (SHEDs), e a possibilidade dessas em combaterem o EO. As DPSCs e SHEDs são células que vêm sendo isoladas desde o inicio dos anos 2000 (GRONTHOS et al., 2000; MIURA et al., 2003). Dentes decíduos e permanentes podem ser facilmente obtidos por motivos ortodônticos, periodontais, cárie e exfoliação natural de decíduos. Esses aspectos corroboram para o conhecimento de que, em curto prazo, a odontologia será a área da saúde com maior possibilidade de aplicações em engenharia tecidual (CHEN et al., 2012). Suscitando, assim, a hipótese de que possa ser também uma grande contribuinte no desenvolvimento de terapias de doenças causadas ou intensificadas pela presença de EO.

Um aspecto decisivo para a realização de estudos laboratoriais e clínicos com SHEDs e DPSCs é que essas células estão presentes em pequenas quantidades, o que torna indubitável a necessidade do crescimento *in vitro* para gerar quantidade suficiente de células a fim de proporcionar avanços práticos na área da saúde (CAPLAN et al., 2009). Dessa forma, torna-se imprescindível tentar recriar *in vitro,* da melhor forma possível, os nichos onde naturalmente as MSCs indiferenciadas estão inseridas e, além disso, determinar o método de isolamento mais adequado para esses tipos celulares. O conhecimento sobre nichos é deverás relevante, haja vista que são

microambientes responsáveis por determinar o destino, a migração e o envelhecimento celular (SCHOFIELD et al., 1978). Estes nichos são altamente especializados e requererem uma organização específica para balancear e controlar a capacidade de autorenovação e proliferação celular (HARADA et al.,2004).

Como supracitado, a investigação do potencial de MSCs para combater o EO ainda é pouco estudada. Portanto, salienta-se e justifica-se assim a execução desse projeto, uma vez que ainda são necessárias mais pesquisas para compreender como esse mecanismo funciona, como por exemplo, esclarecer aspectos sobre a capacidade de tolerância de DPSCs e SHEDs a um ambiente de EO, bem como a possibilidade dessas células reverterem esse ambiente danoso.

2.2 Proposição

2.2.1 Objetivo geral

Isolar e caracterizar as SHEDs e DPSCs e avaliar o seu potencial de modular o estresse oxidativo *in vitro*.

2.2.2 Objetivos específicos

- Realizar uma revisão de literatura sistemática com intuito de analisar os aspectos relevantes das técnicas mais prevalentes para realização do isolamento das SHEDs e DPSCs;
- Isolar as SHEDs e os fibroblastos pulpares;
- Caracterizar as SHEDs e as DPSCs através da técnica de citometria de fluxo;
- Caracterizar as SHEDs e as DPSCs através da diferenciação osteogênica, condrogênica e adipogênica;
- Avaliar os danos oxidativos as SHEDs, as DPSCs e aos fibroblastos pulpares através da determinação de substâncias reativas ao TBARS;
- Avaliar a capacidade antioxidante das diferentes linhagens celulares através da determinação da atividade da CAT;
- Avaliar a capacidade antioxidante das diferentes linhagens celulares através da determinação da atividade da SOD;

- Avaliar a capacidade antioxidante das diferentes linhagens celulares através da determinação da atividade da GPx;

2.4 Materiais e métodos

2.4.1 Revisão Sistemática

2.4.1.1 Estratégias de Busca

A revisão sistemática será realizada buscando artigos nas seguintes bases de dados: Pubmed, Isi, Scopus e Scielo. A proposta consiste em buscar artigos que atendam um único critério de inclusão: estudos que foram utilizadas células tronco de polpa dental de origem humana. As palavras chaves para buscas nas bases de dados serão as siglas referentes às células tronco da polpa de dentes permanentes – DPSCs, e às de dentes decíduos – SHEDs.

2.4.1.2 Seleção de Artigos

Haja vista que o primeiro relato de isolamento de DPSCs na literatura tenha sido no ano 2000 (GRONTHOS et al., 2000) e de SHEDs no ano 2003 (MIURA et al., 2003) haverá a limitação de buscas referente ao ano de publicação dos artigos. Serão incorporados na revisão artigos publicados a partir de 2000 até o presente momento. Artigos que não sejam escritos na língua inglesa serão descartados, bem como, aqueles que sejam revisões de literatura.

Dois revisores independentes farão a primeira seleção de títulos encontrados nas pesquisas. Em caso de discordância, os revisores discutirão e entrarão em um consenso. Se houver alguma dúvida, o artigo deverá ser lido na íntegra a fim de saná-la.

2.4.1.3 Coleta de Dados

Para identificar mais facilmente as variáveis encontradas nos artigos, serão tabeladas algumas informações pertinentes, tais como:

- Autor e ano de publicação;
- Tipo celular;
- Tipo de dente;
- Idade na extração;

- Sexo;
- Armazenamento e forma de transporte;
- Tempo de armazenamento;
- Limpeza da superfície;
- Método de secção do dente;
- Método de remoção do tecido pulpar do interior da câmara;
- Técnicas de isolamento:

Explante, Mecânico e Enzimático, Mecânico, Enzimático (Tipos de Enzima, Quantidade de Enzima, Filtragem);

- Meios de cultivo;
- Suplementação do meio de cultivo;
- Componentes e Quantidade;
- Observações;

2.4.2 Ensaio *in vitro*

2.4.2.1 Descongelamento de DPSCs

As DPSCs foram previamente isoladas e congeladas durante a realização do Trabalho de Conclusão de Curso de Camila Perelló Ferrúa, em 2013/1, intitulado “Levantamento e análise de dados referentes ao isolamento e cultivo de células tronco de tecido pulpar/ Isolamento de células tronco da polpa de dentes permanentes”.

Para a execução das demais metodologias, criotubos serão retirados do interior do botijão de nitrogênio líquido e colocados em banho-maria, até que o conteúdo tome a forma líquida. Em seguida, no interior da capela de fluxo laminar as células serão transferidas ao interior de uma garrafa de cultivo, onde será acrescido o meio de cultivo meio de eagle modificado por Dulbecco (DMEM)/ Ham F12 suplementado com 15% de soro fetal bovino (SFB - Hyclone), 1% de antibiótico - Penicilina, Estreptomicina e Glutamina (Gibco), e 1% de aminoácidos não essenciais (AANE) (Gibco) e então armazenada em estufa de CO_2. Passadas 24 horas, tendo havido adesão celular na parede de fundo da garrafa, será feita a troca do meio de cultivo.

Quando a garrafa de cultivo atingir 80% de confluência será realizado o repique celular. Inicialmente o meio de cultivo será removido do interior da garrafa, as células serão lavadas com 2 mL de PBS em concentração de 1%.

Serão adicionados 2 mL de 0,25% Tripsina-EDTA para a adesão celular e realizado o acompanhamento no microscópio invertido. A ação enzimática será inativada acrescentando 2 mL de meio de cultivo e realizando a ressuspenção do meio para desagregar completamente as células. Em sequência, será adicionado 8 mL de meio, o líquido total será ressuspendido e feita a primeira passagem celular, na razão de 1:2. As garrafas serão, então, levadas para a incubação em estufa sob temperatura de 37°C, em atmosfera úmida e com 5% de CO_2. Nas passagens seguintes passagens, o processo de repique será realizado em razão de 1:3.

2.4.2.2 Isolamento de SHEDs

Serão coletados primeiros molares hígidos, recém extraídos por alunos das disciplinas de Odontopediatria I e II da FO-UFPel (Aprovado no Comitê de Ética em Pesquisa da FO-UFPel, sob o número de protocolo 38/2013). Imediatamente após a exodontia, os dentes serão apanhados pela coroa e limpos com uma gaze estéril embebida em clorexidina 0,2%. Se a coroa estiver hígida serão feitas duas canaletas com ponta diamantada estéril, número 4138 (KG Sorensen), e caneta de alta rotação sob irrigação constante de água milique estéril. Cada canaleta deverá medir aproximadamente 2 mm, sendo uma na face oclusal e outra em uma das faces proximais em direção ao longo eixo do dente (CONDE et al., 2011).

Subsequentemente, os dentes serão armazenados em falcons de 50 mL contendo 15 mL de DMEM acrescido de antibiótico (Cultilab), enriquecido com 10% de SFB e colocados no interior de uma caixa de isopor com gelo para serem transportados ao laboratório. No laboratório os dentes serão retirados, com auxílio de uma pinça estéril, do interior dos falcons e colocados sobre algumas gazes. Se a coroa estiver parcialmente reabsorvida e o tecido pulpar exposto não se faz necessária a criação de canaletas. O tecido pulpar deve ser imediatamente removido da câmara com auxílio de curetas de dentina estéreis. Uma vez que o processo de rizólise não tenha atingido a porção coronária, um cinzel será apoiado sobre a canaleta proximal e com auxílio de um martelo, uma linha de fratura será realizada no dente. Rapidamente, as partes dos dentes serão aproximadas e levadas ao interior da capela de fluxo laminar.

A polpa será removida da câmara pulpar com curetas de dentina estéreis e colocada sobre uma placa de petri plástica, onde haverá 0,5 mL de meio DMEM/ Ham F12, suplementado com 15% de SFB (Hyclone), 1% de antibióticos (Penicilina, Estreptomicina e Glutamina) (Gibco) e 1% de AANE (Gibco). Na placa de petri sob o meio específico para células tronco, usando curetas de dentina e lâmina de bisturi número 15, a polpa será fragmentada em explantes. Em uma placa de 6 poços, serão feitas ranhuras no fundo de cada poço com uma lâmina de bisturi número 15. Os explantes serão transferidos para as placas, de modo a obter-se um explante por ranhura. Serão adicionados 2 mL de meio DMEM a cada poço e as placas incubadas em estufa, por 14 dias, sob temperatura de 37°C, em atmosfera úmida e com 5% de CO_2.

2.4.2.3 Isolamento de fibroblastos pulpares

Serão coletados terceiros molares hígidos, recém extraídos por alunos estagiários do Centro de Especialidades Odontológicas de Cirurgia da Faculdade de Odontologia da Universidade Federal de Pelotas (FO-UFPel) (Aprovado no Comitê de Ética em Pesquisa da FO-UFPel, sob o número de protocolo 38/2013). Imediatamente após a exodontia, os dentes serão apanhados pela coroa e limpos com uma gaze estéril embebida em clorexidina 0,2%. Em seguida, serão feitas duas canaletas com ponta diamantada estéril, número 4138 (KG Sorensen), e caneta de alta rotação sob irrigação constante de água milique estéril. Cada canaleta deverá medir aproximadamente 2 mm, sendo uma na face oclusal e outra em uma das faces proximais em direção ao longo eixo do dente (CONDE et al. 2011). Subsequentemente, os dentes serão armazenados em falcons de 50 mL contendo 15 mL de DMEM acrescido de antibiótico (Cultilab), enriquecido com 10% de SFB (Gibco) e colocados no interior de uma caixa de isopor com gelo para serem transportados ao laboratório. No laboratório os dentes serão retirados, com auxílio de uma pinça estéril, do interior dos falcons e colocados sobre algumas gazes. Um cinzel será apoiado sobre a canaleta proximal e com auxílio de um martelo, uma linha de fratura será realizada no dente. Rapidamente, as partes dos dentes serão aproximadas e levadas ao interior da capela de fluxo laminar. A polpa será removida da câmara pulpar com curetas de dentina estéreis e colocada sobre

uma placa de petri plástica, onde haverá 0,5 mL de meio DMEM, suplementado com 10% de SFB (Gibco). Na placa de petri sob o meio DMEM, usando curetas de dentina e lâmina de bisturi número 15, a polpa será fragmentada em explantes. Em uma placa de 6 poços, serão feitas ranhuras no fundo de cada poço com uma lâmina de bisturi número 15. Os explantes serão transferidos para as placas, de modo a obter-se um explante por ranhura. Serão adicionados 2 mL de meio DMEM a cada poço e as placas incubadas em estufa, por 14 dias, sob temperatura de 37°C, em atmosfera úmida e com 5% de CO_2.

2.4.2.4 Manutenção Celular

Após duas semanas, as placas com SHEDs e fibroblastos isolados serão analisadas em um microscópio invertido TS100 TS100-F da Nikon, onde se observará a migração celular oriunda dos explantes. Um acompanhamento da morfologia e do crescimento celular será realizado através de repetidas análises microscópicas. No momento em que houver cerca de 80% de confluência celular, os explantes serão retirados dos poços com uma pinça e transferidos para garrafas de cultivo de 25 cm^2 de superfície. Em seguida, as células aderidas nas placas serão lavadas com 2 mL de PBS na concentração de 1%. Adicionar-se-á 2 mL de solução 0,25% Tripsina-EDTA (Cultilab) para que as células possam desaderir do poço, com acompanhamento por microscopia óptica. Quando o agregado celular estiver em pequenos grumos, inativar-se-á a ação da tripsina adicionando-se 2 mL de meio de cultivo e ressuspendendo o meio para desagregar completamente as células. Em seguida, a suspensão celular será transferida para uma garrafa de cultura (P0). Colocar-se-á 1 mL de meio de cultivo no poço, que será ressuspendido e transferido para a garrafa, repetindo duas vezes essa etapa e totalizando 6 mL de meio em P0, garantindo a completa remoção das células presentes no poço. A garrafa será transferida para a encubação em estufa, sob temperatura de 37°C, em atmosfera úmida e com 5% de CO_2.

Quando P0 atingir cerca de 80% de confluência, será realizado o repique celular. Inicialmente o meio de cultivo será removido do interior da garrafa, as células serão lavadas com 2 mL de PBS em concentração de 1%. Serão adicionados 2 mL de 0,25% Tripsina-EDTA para a adesão celular e será

realizado o acompanhamento no microscópio invertido. A ação enzimática será inativada acrescentando 2 mL de meio de cultivo, e então será realizada a ressuspenção do meio para desagregar completamente as células. Em sequência, adiciona-se 8 mL de meio, o líquido total será ressuspendido e feita a primeira passagem celular, na razão de 1:2. As garrafas serão, então, levadas para a incubação em estufa sob temperatura de 37°C, em atmosfera úmida e com 5% de CO_2. Nas seguintes passagens, o processo de repique será realizado em razão de 1:3.

2.4.2.5 Caracterização de DPSCs e SHEDs

2.4.2.5.1 Citometria de Fluxo

A análise de citometria de fluxo será realizada em culturas de DPSCs e SHEDs, nas passagens 5 e 7. Uma quantidade de 10^6 células serão incubadas com os seguinte anticorpos conjugados contra aglomerados humanos de diferenciação (CDs) e moléculas de superfície celular: CD29 / PE, CD34 / PE, CD44 / FITC, CD45 / FITC, CD90 / FITC, CD117 / PE, CD133 / PE, CD184 / PE e leucócitos humanos antígeno de histocompatibilidade principal de classe II da superfície celular complexa receptor (HLA-DR) / FITC (Pharmingen-BD Biosciences, San Diego, CA), CD146 e / FITC e estromal marcador de superfície celular 1 (STRO-1) / FITC (Santacruz, Santa Cruz, CA). O isotipo da imunoglobulina G de ratos controles conjugados com PE e FITC serão utilizados para determinar a positividade das amostras. Todas as incubações serão realizadas durante 30 min a 4ºC, e as células lavadas com PBS após cada incubação.

Apenas as células vivas serão analisadas por exclusão de células mortas que forem positivo para o 7-Amino-Actinomicina D (7-AAD) (Invitrogen, Carlsbad, CA) em uma concentração definitiva de 1 mg / mL. As células vivas serão avaliadas por dois marcadores de superfície celular simultaneamente, utilizando anticorpos monoclonais contra os antigenos listados anteriormente. A aquisição de dados será realizada através do citômetro de fluxo FACSCalibur (BD Biosciences), e 10.000 eventos serão analisados utilizando o software CELLQuest (BD Biosciences) (BERNARDI et al., 2011).

2.4.6.2 Diferenciação Osteogênica, Adipogênica e Condrogênica de DPSCs e SHEDs

Para avaliar a capacidade das células para se diferenciarem em outros tipos celulares, serão plaqueadas 10^4 células/cm^2 em placas de 12 poços. Quando as culturas atingirem 70% de confluência, o meio de crescimento será substituído por um meio específico de indução, e as células deverão ser mantidas nos meios específicos de diferenciação de 2 á 4 semanas. Para cada tipo de célula a ser diferenciada, um controlo negativo que consiste em as mesmas células mantidas em meio de cultura convencional.

Para a diferenciação osteogênica, o meio utilizado será DMEM / HEPES suplementado com 10% de SFB; 10% b-glicerofosfato, 10 mmol/L; ácido ascórbico 2-fosfato, 5 mg/ml; e 0,1% de dexametasona, 10^{-5} mol/L.

Para a diferenciação adipogênica, o meio foi de Iscove (Gibco) suplementado com plasma humano a 20%, 10^{-7} mol/L de dexametasona, 2,5 mg/ml de insulina bovina, 5 mmol/L de indometacina, 5 mmol/L de rosiglitazona, 10 UI/ml de heparina de sódio.

Para a diferenciação condrogênica o meio utilizado será DMEM/HEPES, suplementado com 50 nmol/L ascórbico ácido 2-fosfato, 6,25 mg/ml de insulina de bovino, e 10 mg/mL de fator de transformação de crescimento-beta 1 (TGF-β1) (Millipore, Tóquio, Japão). Após o período de diferenciação, as culturas serão lavadas com água desionizada e fixadas em paraformaldeído a 4% de 20 min à 1h. As células que sofrerem diferenciação osteogênica serão coradas com Alizarin Red, as que sofrerem diferenciação adipogênica com Red Oil, e por fim, células que sofrerem diferenciação condrogênica serão coradas com Alcian Blue.

2.4.2.6 Ensaio de Viabilidade Celular

A viabilidade celular das DPSCs, SHEDs e fibroblastos serão determinadas em dois momentos, no processo de caracterização das SHEDs e DPSCs e na avaliação dose resposta dos três tipos celulares mediante a indução de um ambiente de EO. A viabilidade celular será analisada através de um ensaio colorimétrico de MTT (brometo de 3-[4,5-dimetil-tiazol-2-il]-2,5-difeniltetrazólio), onde este composto solúvel é reduzido a cristais insolúveis de formazan (NEDEL et al., 2012).

Para o processo de caracterização celular o ensaio será realizado no momento em que as SHEDs e DPSCs estiverem em P3, P4, P5 e P6 (BERNARDI et al., 2011). Para o ensaio de avaliação do EO serão utilizadas DPSCs, SHEDs e fibroblastos pulpares em P3 e P10, no intuito de avaliar a perda ou o ganho na modulação do EO com o aumento das passagens celulares.

Para ambos os ensaios serão semeadas placas de 96 poços para cada passagem celular, onde serão realizadas 5 repetições, com densidade celular de 10^6 células/poço. Para tanto quando as células atingirem cerca de 80% de confluência, o meio de cultivo celular será descartado e as células lavadas com 3 mL de PBS em concentração de 1%. Serão adicionados 3 mL de Tripsina-EDTA a 0,25% para a desadesão celular e acompanhamento em microscópio invertido. Inativa-se a ação enzimática acrescentando 3 mL de meio de cultivo. Dos 6 mL totais serão removidos 1 mL para a realização da contagem celular, a qual será efetuada utilizando-se o microscópio invertido e a câmara de Neubauer.

Para o ensaio de caracterização das SHEDs e DPSCs as células serão avaliadas após 1, 3, 5 e 7 dias de cultivo. Para o ensaio de EO o meio de cultivo deverá ser suplementado com H_2O_2 (Sigma) e mantido em contato com as DPSCs, SHEDs e fibroblastos pulpares por diferentes períodos de tempo e concentrações, a fim de estabelecer o tempo e a concentração letal de H_2O_2. Devido à escassez de artigos na literatura que tenham estimulado EO em células tronco pulpares, a concentração de H_2O_2 será determinada através de uma curva dose-resposta. Quanto ao tempo de estimulação do insulto, as células serão mantidas em contato com H_2O_2 por no máximo 18 h (VALLE-PRIETO et al., 2010), tempo intermediário experimental de 10 e mínimo de 2 h (BORODKINA et al., 2014; VINOTH et al., 2014).

Então aos experimentos, tanto de caracterização como de estresse oxidativo, serão adicionados aos 200 µL de meio de cultivo preexistentes, 20 µL de MTT (5 mg de MTT/mL de meio de cultivo) por poços, o qual será mantido em contato com as células por 4 h. Em seguida, o líquido contido nos poços será desprezado, adiciona-se 200 µL de DMSO e coloca-se as placas em um *shaker* por 5 min a 150 rpm. Realiza-se então a leitura da absorbância

em espectofotômetro (Thermo Plate TP-Reader) em um comprimento de onda de 450 nm.

2.4.2.7 Avaliação do Dano Oxidativo e da Atividade das Enzimas Antioxidantes

2.4.7.1 Determinação de Substâncias Reativas ao TBARS

Será realizada pelo método de Esterbauer e Cheeseman. As amostras reagirão com 10% de ácido tricloroacético e 0,67% de ácido tiobarbitúrico e em seguida serão aquecidas em banho seco por 1 hora. A curva de calibração será realizada utilizando 1,1,3,3-tetrametoxipropano, seguindo o mesmo tratamento das amostras. A absorbância de TBARS será determinada em 535 nm. Os resultados serão calculados em nmol de TBARS/mg de proteína (ESTERBAUER e CHEESEMAN, 1990).

2.4.7.2 Determinação da Atividade da CAT

Será determinada de acordo com o método descrito por Aebi (1984), baseado na decomposição da H_2O_2, acompanhada a 240 nm, à temperatura ambiente. Os resultados serão expressos em unidades de atividade de catalase (sendo uma unidade definida como a quantidade de enzima que decompõe 1 µmol de H2O2/min/mg de proteína (AEBI et al., 1984).

2.4.7.3 Determinação da Atividade da SOD

O método utilizado será realizado conforme descrito por Misra and Fridovich (1972). O método baseia-se na inibição de superóxido dismutase dependente da auto-oxidação de adrenalina em um comprimento de onda de 480 nm. Uma unidade de atividade de superóxido dismutase é definida como a quantidade necessária para reduzir a velocidade da reação em 50%. Os resultados serão expressos em U/mg de proteína (MISRA e FRIDOVICH, 1972).

A concentração de proteína será determinada pelo método de Lowry utilizando albumina bovina como padrão. O princípio do método de Lowry baseia-se numa mistura contendo molibdato tungstato e ácido fosfórico, (reagente Folin-Ciocalteau), que sofre uma redução quando reage com

proteínas, na presença do catalisador cobre (II), e produz um composto com absorção máxima em 750 nm (LOWRY et al., 1951).

2.4.7.4 Determinação da atividade da GPX1

Para quantificar a atividade de GPX1, 1 mL da solução de lise tampão de GPX1 (50 mM Tris-HCl, 5 mM EDTA, e 1 mM de DTT, pH 7,5) será utilizada para extrair proteínas a partir de 1 x 10^6 de células. A atividade GPX1 será medida utilizando kit de ensaio dos peróxidos glutationa (Calbiochem). Este método permitirá a quantificação da atividade da GPX1 através de espectofotômetro em um intervalo de 50 - 344 nmol/(min mL) (NADPH oxidado).

2.4.8 Reação em Cadeia da Polimerase em Tempo Real (qPCR)

O ácido ribonucléico (RNA) total dos diferentes tipos celulares será extraído utilizando reagente TRIzol (Invitrogen, Carlsbad, CA), seguindo as indicações do fabricante. O DNA genômico será degradado utilizando DNAase I (Invitrogen). O RNA será quantificado espectrofotometricamente a 260 nm. Para reação de transcrição reversa, 1 mg do RNA total, 200 U M-MLV de transcriptase reversa (Promega, Madison, WI), e 300 pmol de oligo(dT) primer serão utilizados para 25 ml de mistura da reação total. A reação será conduzida seguindo as instruções do fabricante da enzima. O PCR em tempo real será realizado usando 2 µL de reação de TR, 0,5 mM de cada um dos primers específicos (Tabela 1), e DNA-LightCycler SYBR Green I Kit (Roche, Indianapolis, IN), em um termociclador PCR em tempo real no termociclador 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, EUA). (Applied Biosystems, Foster City, CA, EUA). O nível de expressão de cada uma das enzimas será padronizado contra GAPDH e a comparação entre as células pelo método comparativo Ct (SCHIMITTGEN, et al., 2008).

Gene	Sentido (5' – 3')	Anti sentido (3' – 5')	Amplicons	
			Temp(°C)	Tamanho(bp)
hSOD1	GGT CCT CAC TTT AAT CCT CTA T	CAT CTT TGT CAG CAG TCA CAT T	83	96
hSOD2	TGA CAA GTT TAA GGA GAA GC	GAA TAA GGC CTG TTG TTC C	85	148
hCAT	TTA ATC CAT TCG ATC TCA CC	GGC GGT GAG TGT CAG GAT AG	87	210
hGPX1	CGC CAC CGC GCT TAT GAC CG	GCA GCA CTG CAA CTG CCA AGC AG	93	238
GAPDH	CAG CCT CAA GAT CAT CAG CA	CAT GAG TCC TTC CAC GAT AC	85	100

Tabela 1: Primers específicos para Reação em Cadeia da Polimerase em Tempo Real (Adaptada de VALLE-PRIETO et al., 2010).

2.4.9 Análise de Dados

A avaliação dos dados será realizada utilizando a Análise de Variância (ANOVA) de duas vias seguido pelo teste de Tukey para comparações múltiplas, com nível de significância de $p<0,05$. Para a elaboração dos gráficos será utilizado o Programa GraphPad Prism, versão 4.00 para Windows (GraphPad Software, San Diego, USA).

2.5 Orçamento

1,1,3,3- Tetrametoxipropano, Sigma Aldrich, 100 ml	142,00
Ácido Tricloroacético, Synth, 250 g	163,39
Albulmina Bovina Liofilizada, Sigma Aldrich, 1 g	248,00
Alcian Blue, Sigma Aldrich, 250 ml	284,00
Alizarin Red, Sigma Aldrich, 25 g	296,00
Catalase, Sigma Aldrich, 1 g	125,00
Dexametasona, Sigma Aldrich, 25 mg	152,00
DMEM, Cultilab, 500 ml	149,00
DMEM/ Ham F12, 500 ml	182,00
DMSO, Synth, 1000 ml	53,38
Epinefrina, Sigma Aldrich, 5 g	389,00
Falcon 15 ml, 100 unidades	42,00
Falcon de 50 ml, 50 unidades	35,00
Insulina bovina, Sigma Aldrich	534,00
Materiais para Citometria de Fluxo	6.000,00
Materiais para qPCR	1.491,21
MTT, Sigma Aldrich, 250 mg	164,00
PBS, Gibco, 500 ml	310,00
Ponta diamantada KG Sorense, 4138	8,86
Reagente Folin Ciocalteu, Sigma Aldrich, 100ml	225,00
Red Oil, Sigma Aldrich, 25 g	107,00
Soro Fetal Bovino, Gibco, 500 ml	294,00
Sulfato de Cobre, Synth 100 g	25,57
TGF-beta 1, Sigma Aldrich, 5UG	649,00
Tripsina/EDTA, Cultilab, 500ml	329,00
Total	**14.912,42**

2.6 Cronograma

	Atividade	Mês																		
		Mar - Ago /14	Set/ 14	Out /14	Nov /14	Dez /14	Jan /15	Fev /15	Mar /15	Abr/ 15	Mai /15	Jun /15	Jun /15	Ago /15	Set/ 15	Out /15	Nov /15	Dez /15	Jan /16	Fev /16
F 1	Atualização bibliográfica	x	x	x	x	x	x	x	x	x	x	x	x	x						
	Aquisição de materiais e equipamentos	x	x																	
	Realização de experimento-piloto			x	x															
F 2	Execução do projeto					x	x	x	x	x	x	x								
	Coleta de dados referentes á revisão de literatura					x	x	x	x	x	x	x								
	Tabulação dos dados e análise estatística												x	x						
	Apresentação de resultados finais em congresso														x	X				
F 3	Redação de artigos científicos																x	x	x	
	Defesa																			**x**

3 Relatório do trabalho de campo

Venho por meio do relatório do trabalho de campo da referida dissertação, justificar as alterações presentes entre o projeto de qualificação proposto e os artigos produzidos ao fim desse processo. Inicialmente, no que tange a proposta de realização de uma revisão sistemática, o objetivo consistia em analisar os aspectos relevantes das técnicas mais prevalentes para realização do isolamento das SHEDs e DPSCs. No entanto, destaca-se que devido ao volume de dados obtidos ao fim da análise dos mesmos, bem como frente à dificuldade de discussão devido a grande quantidade de dados coletados e no intuito de não subjugar as informações obtidas, considerando os dados, uns mais relevantes em detrimento á outros, fez-se necessária a redação de um segundo artigo de revisão sistemática. Assim, ficou estabelecido o artigo 1 referente a forma como as técnicas de isolamento de DPSCs vem sendo conduzidas ao longo dos últimos 15 anos, desde que foram isoladas pela primeira vez. Não menos relevante, o artigo 2, constituiu-se dos aspectos em relação ao manejo da estrutura dental previamente ao processo de isolamento celular, propriamente dito.

No que diz respeito à proposta referente ao ensaio laboratorial, que consistiu em isolar, caracterizar e verificar o potencial de modulação do estresse oxidativo, de fibroblastos pulpares, DPSCs e SHEDs, vale destacar que a mesma foi ligeiramente alterada. Primeiramente, destaca-se que algumas modificações em relação ao proposto foram geradas em consequência da necessidade de atecipar a data de defesa da dissertação em torno de 4 meses, com intuito de que fosse possível a inscrição da candidata, num processo seletivo de doutorado em outra instituição. Sendo assim, inicialmente fora proposta a verificação da atividade das enzimas SOD, CAT, GPX e determinação quantitativa das substancias reativas ao TBARS. Já foi realizada a aquisição de todos os reagentes necessários para efetuar as técnicas supracitadas, e até o presente momento realizou-se os testes iniciais da atividade da enzima SOD. É importante salientar que as amostras referentes as DPSCs, as SHEDs e aos fibroblastos pulpares insultadas com peróxidos de hidrogênio (H_2O_2) já encontram-se congeladas aguardando para serem realizadas as avaliações das enzimas CAT, GPX e

a determinação quantitativa das substancias reativas ao TBARS. No que diz respeito a realização da expressão gênica, o nosso grupo de pesquisas detém verba destinada á aquisição desses kits, contudo os mesmos ainda não foram realizados em detrimento a necessidade de antecipação da defesa.

É imprescindível mencionar que algumas adaptações metodológicas, fizeram-se necessárias, haja vista os cortes no orçamento do Estado destinados á pesquisa no Brasil. O processo de caracterização das células tronco obtidas nesse estudo fora proposto, no entanto, vale destacar que as adaptações sofridas consistem na realização de parte das técnicas de caracterização, anteriormente propostas. Primeiramente, foram realizados ensaio de viabilidade celular a fim de analisar a taxa de proliferação dessas células, no intuito de averiguar se os níveis de proliferação celular são compatíveis com os que já se tem pré-estabelecido na literatura. Além disso, verificou-se a capacidade de diferenciação celular em tecido osteogênico e os resultados parecem animadores, contudo os demais ensaios de diferenciação propostos foram, momentaneamente, suspensos. O grupo revela que tem realizado contatos com outras universidades, a fim de alicerçar parcerias futuras para a realização desses ensaios.

Nesse contexto de corta de verbas, aliado ao fato de que a literatura não vem realizando, repetidamente, o processo de caracterização para cada elemento dental fonte de células tronco, bem como devido ao fato de que membros do nosso grupo de pesquisa realizaram curso prático de isolamento de DPSCs e SHEDs no Instituto Butantan, onde as células obtidas pela metodologia, que repetimos na Universidade Federal de Pelotas, são comprovadamente células tronco propriamente ditas, via ensaio com citometria de fluxo, justifica-se a realização de somente dois testes para a caracterização celular – ensaio de viabilidade celular e diferenciação osteogênica.

4 **Artigo 1**

How has DPSCs isolation been conducted? A systematic review

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Abstract

Currently stem cells have been used as a potential tool for improving the treatment of various diseases. In this context, dental pulp stem cells (DPSCs) have been considered a promising alternative, however there is a gap in the literature towards its isolation. Thus, this study aimed to obtain the profile of DPSCs isolation protocols and analyze the possible risk factors that could change the native behavior of these cells. The electronic search was conducted without initial date restriction up to and including (April 2014) in PubMed, Scopus, Scielo and ISI Web of Knowledge databases 222 articles were included and the information analysis was performed concerning the following items: author's name, year of publication, DPSCs isolation technique [explant, enzymatic, mechanic technique (particularities of each technique, such as size of the explant, contact time and enzyme concentration used, cell filtration, tissue fragmentation method)], culture medium, medium supplementation (components and concentration), as well as relevant observations. In general, it was observed that the enzymatic cell isolation technique is the most used technique to isolate DPSC, and collagenase type I and dispase the most used enzymes in the concentrations of 3 mg / ml + 4 mg/ml, respectively. When the filtering step was performed 40 µM was the preferably pore size is. The α-MEM, penicillin, streptomycin, L-glutamine and SFB are the most recomende for DPSCs cultivation. In conclusion over the past 15 years many studies have been conducted using DPSCs, however this is the first systematic review regarding the isolation of DPSCs. The isolation of DPSCs showed great variability, hampering the development of standard protocols to achieve DPSCs *in vitro* with similar characteristics from those *in vivo*, and possibly influencing the results of the same evaluated outcomes. Finally, based in the results from the systematic review we proposed a standard protocol for DPSC isolation.

Keywords: Dental pulp stem cell; Isolation; Dental pulp; Stem cells.

Introduction

Currently mesenchymal stem cells (MSCs) are commonly investigated for their potential to develop areas such as cell therapy, tissue engineering and molecular biology, optimizing new treatment alternatives for diseases that affect and are common worldwide (Uccelli, Moretta et al. 2008). MSCs can be related to positive results in orthopedics treatments (Khan, Longo et al. 2012), autoimmune diseases (Uccelli, Moretta et al. 2008, De Miguel, Fuentes-Julian et al. 2012), inflammatory process (Chang, Zhang et al. 2005). In addition, their benefits have been noted in diseases related to oxidative stress, as acute myocardial infarction, ischemic stroke and diabetes, through enzymatic pathway (Valle-Prieto and Conget 2010) and by paracrine activity (Liu, McTaggart et al. 2012).

In 2000, Gronthos and colleagues identified MSCs in the pulp of permanent teeth: the dental pulp stem cells (DPSCs) (Gronthos, Mankani et al. 2000). This discovery provided a new source of stem cells, since DPSCs can be obtained by a minimally invasive process (Liu, Yu et al. 2015), from tooth extracted due to orthodontic reasons, periodontal disease or caries (Chen, Sun et al. 2012). Furthermore, DPSCs have high proliferative capacity, holding the potential of self-renewal and differentiation into multiple cell lineages (Gronthos, Mankani et al. 2000).

In this context, several studies have credited the use of DPSCs as an appropriate cell model for various applications in different areas of health. It is known that through the use of recombinant transforming growth factor β3 (TGF-β3) DPSCs can differentiate into a chondrogenic lineage, in similarity to tissue *in vivo* (Rizk and Rabie 2013). Due to their high osteogenic potential, DPSCs have shown the capacity to form bone around dental implants (Ito, Yamada et al. 2011), bone resorption (D'Aquino, Schirra et al. 2007), and resections resulting from surgery to remove tumors (Yelick and Vacanti 2006). Another important aspect suggests that DPSCs can inhibit the proliferation of T cells, which modulate the immune response from recently transplanted patients (Pierdomenico, Bonsi et al. 2005).

Since DPSCs knowledge advanced more rapidly towards the final goal, clinical application, some concerns, such as stem cell isolation, seem to be relegated to

the background of basic science. Despite the use of DPSCs in the last 15 years and there promising future, there is a gap in the literature towards their isolation. Frequently authors report inconsistent methodologies with mislaid information, hampering the search for standard protocols to achieve *in vitro* DPSCs isolation with similar characteristics from those *in vivo*, and therefore possibly influencing the results of the same evaluated outcomes. Thus, the aim of this study was to systematically review the literature in order to identify the profile of DPSCs isolation protocols and analyze the possible risk factors that could change the native behavior of these cells.

Methods

- Review questions

In the literature, how has the isolation of DPSCs been conducted?
Is there a standardized protocol in the literature for DPSCs isolation?

- Inclusion and exclusion criteria

The inclusion criteria for article assessment were: description of stem cell isolation from human dental pulp of permanent teeth. Exclusion criteria were: literature reviews, cells from non human source, stem cells from other sources than the pulp, isolation technique not described, human cells but not stem, SHEDs, congress summary, patents, book section, hypothesis articles, editorial, letters to the editor, news, protocols, withdrawn, interview, articles which are not written in the English language and articles that were not fully available and/or after e-mails sent to the authors there was no reply. Articles could be excluded through more than one exclusion criterion.

- Search strategy

The electronic search was conducted without initial date restriction up to and including (April 2014) in PubMed, Scopus, Scielo and ISI Web of Knowledge databases in order to identify studies that demonstrate the methodology used for DPSCs isolation. An initial search was conducted using the following MeSH terms: “(dental pulp stem cell [MeSH])”; “(dental pulp [MeSH])” AND “(stem cell [MeSH])”; “(“dental pulp stem cell” [MeSH]”)”. No language and date restrictions were applied in the searches.

All references were managed in the EndNote X7 software (Thomson Reuters, New York, NY, US). Initially, duplicate references were excluded. Titles, abstracts and studies methodologies were screened based on the inclusion and exclusion criteria by two reviewers independently (CPF and EGZC). Lists were compared and in case of disagreement, a consensus was reached by discussion. When a consensus was not achieved, a third reviewer decided if the article should be included (FN). This systematic review followed the PRISMA statements (Moher, Liberati et al. 2009) with some adjustments (Figure 1).

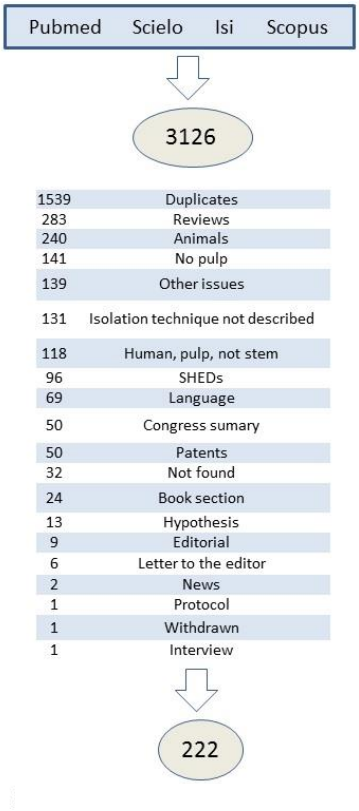


Figure 1 – Flowchart with the studies selection process for inclusion in the systematic review (Exclusion reasons: a study could have fulfilled more than one criterion).

- Data extraction and quality assessment

After screening, the following data was collected from the articles: author's name, year of publication, DPSCs isolation technique [explant, enzymatic, mechanic technique (particularities of each technique, such as size of the explant, contact time and enzyme concentration used, cell filtration, tissue fragmentation method)], culture medium, medium supplementation

(components and concentration), as well as relevant observations. Authors were contacted in order to clarify any queries on the study methodology or result. Data were extracted and tabulated independently by two reviewers (CPF and LCdaR) to be submitted to a descriptive analysis. Cases of disagreement were discussed until a consensus was reached. When a consensus was not obtained, a third reviewer participated in the discussion (FN).

- Assessment of risk of bias in included studies

Risk of bias was evaluated according to the articles description of the following parameters for study quality assessment: (a) size of explant fragment in the explant technique, (b) culture medium, (c) cell filtration in the enzymatic technique, (d) serum, (e) medium supplementation, except for serum and (f) enzyme used in enzymatic technique and (g) instrument used for mechanical dissociation in the association technique. If the authors reported the parameters, the article had a “Y” (yes) on that specific parameter; if it had the parameter, if the information was not described, the article received a “N” (no). These data were tabulated and it was established that the presence of "Y" in studies of up to 33.3% represents high risk of bias, if the amount studies represented by "Y" is above 33.3% and even 66.6 % represents average risk, whereas above 66.6% is low risk of bias.

- Impact factor analysis

The impact factor analysis, of the included articles, was performed searching each journal for its impact factor in the year that the corresponding study was published. Then these values were categorized as follows: From 0 to 1, 1.01 to 2, 2.01 to 3, 3.01 to 4, 4.01 to 5, 5.01 to 6, 6.01 to 7, 7.01 to 8, 8.01 to 9, 9.01 to 10, more than 10. Subsequently, a relation between the impact factor and the presence or absence of the following items was established - a) size of explant fragment, (b) culture medium, (c) cell filtration, (d) serum, (e) medium supplementation, except for serum and (f) enzyme used in the enzymatic technique and (g) instrument used for mechanical dissociation in the mechanic technique.

Results

- Descriptive Analysis

Electronic search revealed 3,126 articles. From those 1,539 were duplicated and therefore, removed. A total of 1,587 articles were included for title, abstract and methodology screening. Figure 1 shows flow-chart of the study selection. From those 222 were included for full text analysis. Tooth donor profile is predominantly males (55.9%) with an average age ranging from 17.5 years to 30.6 years.

From the studies included in this review, 100% described the enzyme type. The second item most frequently mentioned by the authors was the culture medium used in DPSCs culture *in vitro*, (95% of the studies). However, the size of explant fragments in the explant technique and cell filtration in the enzymatic and/or mechanical technique were the most neglected ones, with 45.5% and 52.3% of articles respectively not informing this procedure (Table 1).

	Yes		No	
	N	%	N	%
Size of explant fragments	12	54.5%	10	45.5%
Enzyme type	200	100%	0	0%
Enzyme concentration (enzymatic technique)	116	90.6%	12	9.4%
Enzyme concentration (association technique)	67	89.3%	8	10.7%
Culture medium	212	95%	10	5.0%
Cell filtration	106	47.75%	116	52.25%
Serum	195	87.8%	27	12.2%
Supplementation (serum excluded)	179	90.%	20	10%

Table 1. Distribution according to the presence or absence of important aspects of DPSCs isolation.

There are three main techniques that have been described in the literature to isolate DPSCs: explant, enzymatic and mechanical, or in some cases the association between the two mentioned methodologies. Among the 222 selected articles for this systematic review, more than 50% conducted the enzymatic technique, followed by an association between the enzymatic and mechanical technique, representing 33.5%. The isolation techniques less preconized were the explant and mechanical methodologies (Table 2).

	N	%
Enzymatic	126	56.3%
Association (enzyme + mechanical)	75	33.5%
Explant	22	9.82%
Mechanical	1	0.5%
Total	224	100.0%

Table 2. Distribution according to the technique used for DPSCs isolation.

In regard to the enzymatic technique for DPSCs isolation, the literature revealed twenty different combinations of enzymes. Table 3 shows that collagenase type I associated with dispase is the most frequently used, representing more than 54% of the total. The second most frequent type employed is the enzyme collagenase type I alone, which represents 17.2%. Ten different enzyme combinations represent less than 5% of the selected articles (Table 3).

	N	%
Collagenase type I and dispase*	111	54.4%
Collagenase type I	35	17.2%
Collagenase and dispase*	14	6.9%
Collagenase*	9	4.4%
Collagenase type I and dispase type II	8	3.9%
Trypsin	5	2.5%
Collagenase type I and collagenase type II	4	2.0%
Collagenase type IA	2	1.0%
Collagenase type II	2	1.0%
Dispase type I	1	1.0%
Collagenase type A	1	1.0%
Collagenase type IA and dispase*	1	1.0%
Collagenase type II and dispase type I	1	1.0%
Collagenase and dispase type II*	1	1.0%
Collagenase/DNAse	1	1.0%
Collagenase type I and DNAse	1	1.0%
Trypsin and collagenase	1	1.0%
Collagenase Blend type H	1	1.0%
Collagenase type I, colLagenase type II, termolisina	1	1.0%
Not described types of enzymes used	3	1.5%
Total	203	100.0%

* The type of collagenase and/or dispase was not described.

Table 3. Distribution according to the type of enzymes used for DPSCs isolation with the enzymatic technique or in the association of the enzymatic and mechanical techniques.

In the most used combination of enzymes (collagenase type I and dispase) for DPSC isolation with the enzymatic technique or the association of the enzymatic and mechanical techniques, the standard concentration used was, respectively, 3 mg/mL and 4 mg/mL. However, more than seven different concentrations are mentioned for this combination of enzymes and 0.5% of the studies did not describe the quantity used (Table 4).

	N	% Group	% Total
Collagenase type I and dispase			
3 mg/mL + 4 mg/mL	100	90.1%	49.0%
4 mg/mL + 2 mg/mL	3	2.7%	1.5%
0,3 mg/mL + 0,4 mg/mL	2	1.8%	1.0%
2 mg/mL + 4 mg/mL	1	0.9%	1.0%
3 mg/mL + 5 mg/mL	1	0.9%	1.0%
1 mg/mL + 2,4 mg/mL	1	0.9%	1.0%
3 mg/mL + 2,4 mg/mL	1	0.9%	1.0%
0,3% + 0,4%	1	0.9%	1.0%
Used (not described both)	1	0.9%	1.0%
Subtotal	110	100.0%	54.4%
Collagenase type I			
3mg/mL	24	68.6%	11.8%
1mg/mL	3	8.6%	1.5%
2mg/mL	2	5.7%	1.0%
0.1%	1	2.9%	0.5%
1%	1	2.9%	0.5%
Used (not described both)	4	11.4%	2.0%
Subtotal	35	100.0%	17.2%
Collagenase and dispase			
3mg/mL + 4mg/mL	2	14.3%	1.00%
0,2mg/mL + 2mg/mL	1	7.1%	1.00%
1mg/mL + 3mg/mL	1	7.1%	1.00%
2mg/mL + 1mg/mL	1	7.1%	1.00%
Collagenase not described + 2mg/mL	1	7.1%	1.00%
Used (not described both)	8	57.1%	3.9%
Subtotal	14	100.0%	6.9%
Collagenase			
2mg/mL	4	44.4%	1.96%
0.1%	1	11.1%	0.5%
0.2%	1	11.1%	0.5%
0,5mg/mL	1	11.1%	0.5%
625 U/mL	1	11.1%	0.5%
Used (not described)	1	11.1%	0.5%
Subtotal	9	100.0%	4.4%
Collagenase type I and dispase type II			
3mg/mL + 4mg/mL	4	50.0%	2.00%
3mg/mL + 2,4mg/mL	3	37.5%	1.5%

0,3% + 0,1%	1	12.5%	0.5%
Subtotal	8	100.0%	3.9%
Trypsin			
0.05%	2	40.0%	1.0%
3 mL of 0.25%	2	40.0%	1.0%
0.2%	1	20.0%	0.5%
Subtotal	5	100.0%	2.5%
Collagenase type I and collagenase type II			
Used (not described both)	4	100.0%	2.0%
Subtotal	4	100.0%	2.0%
Collagenase type IA			
3mg/mL	2	100.0%	2.0%
Subtotal	2	100.0%	2.0%
Collagenase type II			
0.075%	1	50.0%	0.5%
0.2%	1	50.0%	0.5%
Subtotal	2	100.0%	1.0%
Dispase type I			
Used (not described)	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase type A			
2mg/mL	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase type IA and dispase			
3mg/mL + 4mg/mL	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase type II and dispase type I			
5mg/mL + 2,5mg/mL	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase and dispase type II			
Used (not described both)	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase/DNAse			
4mg/mL + 0,5mg/mL	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase type I and DNAse			
1mg/mL + 25µg/mL	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase Blend type H			
0,5mg/mL	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Collagenase type I, colLagenase type II, termolisina			
Used (not described any)	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Trypsin and collagenase			
Used (not described both)	1	100.0%	0.5%
Subtotal	1	100.0%	0.5%
Total	201		100.0%

Table 4. Distribution according to the combination and concentration of enzymes used for DPSCs isolation with the enzymatic technique or in the association of the enzymatic and mechanical techniques.

When considering DPSCs isolation using the explant technique, an important variable is the size of tissue fragments. Although studies bring up five different sizes, all articles reported sectioning the tissue in small dimensions. Thus, 18.2% stated that fragments were small without mentioning, however, the precise dimension, and 45.5% do not mention the size of the fragments (Table 5).

	N	%
Small fragments	4	18.2%
1–2 mm^3	3	13.6%
1 mm^3	2	9.1%
0.5-1 mm	1	4.6%
1x1x2 mm^3	1	4.6%
60 mm	1	4.6%
Not described the size of fragments	10	45.5%
Total	22	100.0%

Table 5. Distribution according to the size of fragments used in the explant technique.

When the mechanical technique is chosen, either alone or in combination with the enzymatic technique, it is commonly observed the presence of a filtering cell step. Data suggest that 47.8% of authors mention the implementation of this step. Within this group, 69.8% used a 70 µm filter, 10.4% used 100 µm filter and 10.4% used 40 µm filter. Furthermore 9.4% of the studies that mention the filtration technique did not describe the pore size used (Table 6).

	N	%
70 µm	74	69.8%
100 µm	11	10.4%
40 µm	11	10.4%
Not described the size	10	9.4%
Total	106	100.0%

Table 6. Distribution according to the pore size of filters used in the mechanical and in the combination with the enzymatic technique.

Data showed that 95.5% of all studies described the medium used for DPSCs culture (Table 1). Nineteen different types of medium for DPSCs culture *in vitro*, were described. The most commonly used medium was α-MEM (52.3%), followed by DMEM (21.1%) (Table 7).

	N	%
α-MEM	114	52.3%
DMEM	46	21,1%
DMEM/F12	9	4.1%
DMEM-KO	7	3.2%
Low glucose DMEM	7	3.2%
MEM	7	3.2%
MSCM	5	2.3%
Mega Cell	5	2.3%
MCM	3	1.4%
A-DMEM	2	0.9%
DMEM/Ham'sF12	2	0.9%
EBM2	2	0.9%
MCDB-201	2	0.9%
MEM-Earle	2	0.9%
BME	1	0.5%
High-glucose DMEM	1	0.5%
Low glucose DMEM and MCDB-201	1	0.5%
NH stem cell expansion	1	0.5%
Phenol red free L-DMEM	1	0.5%
Total	218	100.0%

Table 7. Distribution according to the types of medium used for culturing DPSCs. Abbreviations: α-MEM - α-minimum essential medium; DMEM - Dulbecco's modified Eagle's medium; DMEM/F12 - Dulbecco's modified Eagle's medium/F12; DMEM-KO - Dulbecco's modified Eagle's medium – knock-out; MEM - Minimum Essential Medium; MSCM - Mesenchymal Stem Cell Medium; MCM - Mesencult Complete Medium; A-DMEM - Advanced Dulbecco's modified Eagle'S medium; DMEM/Ham'sF12 - Dulbecco's modified Eagle's medium/Ham's F12; EBM2 - Endothelial cell basal medium 2; MEM-Earle - Minimum Essential Medium-Earle; BME - Basal Medium Eagle; NH stem cell expansion – Nonhematopoietic stem cell expansion.

The supplementation of DPSCs *in vitro* is also an important issue. In the total studies reviewed 90.0% mentioned the use of supplementation, disregarding serum (Table 1). In relation to serum, the results showed that the use of bovine serum is the most common, followed by calf and human serum, respectively 74.4%, 23.1% and 2.0% of total selected papers. The vast majority of studies supplement the culture medium with two, three or four components, other than serum. The most cited ones are penicillin and streptomycin accounting for more

than 50.0% of all compounds used for supplementation. Besides these, 32 different types of supplements were cited (Table 8).

	N	%
Penicilin	157	26.0%
Streptomicin	151	25.0%
L -glutamine	61	10.1%
L-ascorbic acid	40	6.6%
2P-ascorbic acid	28	4.5%
Glutamine	18	3.0%
Amphotericin	16	2.6%
AA	14	2.3%
Dexamethasone	13	2.1%
Fungizone	12	2.0%
Gentamicin	11	1.8%
PDGF	9	1.5%
Glutamax	9	1.5%
EGF	9	1.5%
Antibiotics	7	1.2%
Insulin	7	1.2%
Selenium	7	1.2%
Transferrin	7	1.2%
hPDGF BB	3	0.5%
LA-BSA	3	0.5%
Glucose	3	0.5%
βFGF	2	0.3%
BSA	2	0.3%
hLIF	2	0.3%
Clarithromycin	2	0.3%
CDLC	2	0.3%
Kanamycin	2	0.3%
Vitamin C	2	0.3%
IGF	1	0.2%
ß-ME	1	0.2%
Ciprofloxacin	1	0.2%
Sodium pyruvate	1	0.2%
Hepes	1	0.2%
Mesenchymal cell growth supplement	1	0.2%
Total	605	100.0%

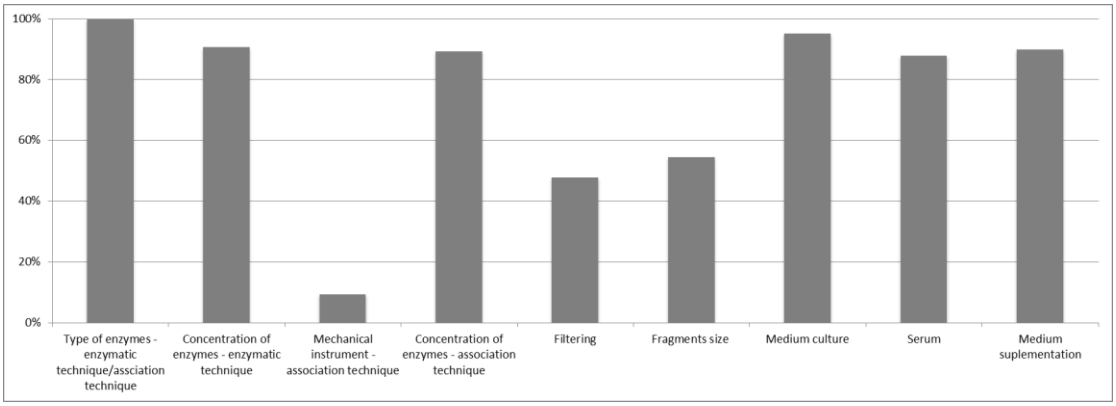
Table 8. Distribution according to the supplementation used in DPSCs culture medium preparation. Abbreviations: AA - antibiotic antimycotic solution; PDGF - platelet-derived growth factor; EGF - epidermal growth factor; hPDGF BB – human recombinant platelet-derived growth factor; LA-BSA - linoleic acid-bovine serum albumin; bFGF - basic fibroblast growth factor; BSA – bovine serum albumin; hLIF – human leukemia inhibitor factor; CDLC - Chemically Defined Lipid Concentrate; IGF - insulin-like growth factor; ß-ME - ß-mercaptoethanol.

	N	%
FBS	148	74.4%
FCS	46	23.1%
HS	4	2.0%
FCS and FBS	1	0.5%
Total	200	100.0%

Table 9. Distribution according to the serum used in DPSCs culture medium preparation. Abbreviations: FBS – Fetal bovine serum; FCS – Fetal Calf Serum; HS – Human Serum.

- Risk of Bias

From 222 studies included in this review, only 9.3% presented the description of the instrument with that mechanical dissociation was performed, in the association technique, representing a high risk of bias. When analyzing the risk of bias over the filtering and fragment size of explant, both showed medium risk of bias, these items were mentioned respectively in 47.8% and 54.6% of all studies. All studies showed the type of enzyme used, indicating a low risk of bias for this variable. In the association technique and the enzymatic technique, the enzyme concentration was described in 89.3% and 90.6% of the studies, respectively. The medium culture (95.1%), serum (87.8%) and the medium supplementation (90.0%) were considered at low risk of bias (Graph 1).



Graph 1. Risk of bias considering aspects reported in the material and method section.

- Standard protocol for DPSCs isolation

Based on the frequency in each step of DPSCs isolation obtained in this systematic review we developed a standardized protocol (Figure 2). However it is important to highlight that this protocol is based on frequency analysis and not in effectiveness of DPSCs isolation.

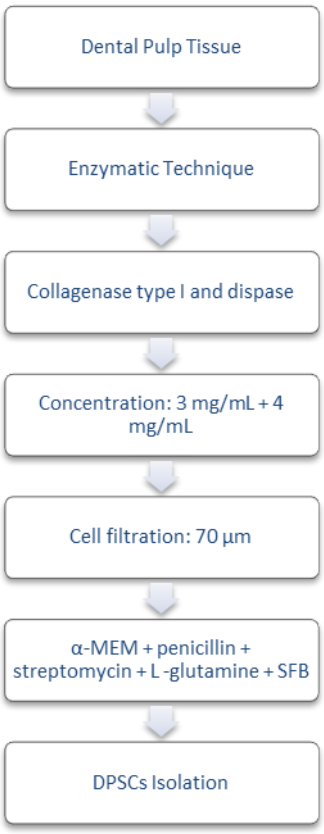


Figure 2 – Flowchart with the most commonly used protocol for DPSCs isolation.

- Impact factor

Of the 222 studies included in this review 153 were published in journal with impact factor lower than 5 and 23 articles were published in journal with impact factor higher than 5. The variable type of enzyme, used in the enzymatic technique and association technique for cell isolation, was present in 100% of the articles regardless of the journal's impact factor where the studies were published. Most of the considered variables were present in a higher relative frequency in the group with impact factor higher than 5. Thus including the enzyme concentration in the enzymatic technique, filtering, fragment size, serum and medium supplementation. In the group published in journal with impact factor lower than 5, higher relative frequency was observed in the variables enzyme concentration in the association technique and medium culture.

Impact Factor	Enzyme type (%)		Enzyme concentration (enzymatic technique) (%)		Enzyme concentration (association technique) (%)		Filtering (%)		Fragments size (%)		Medium culture (%)		Serum (%)		Medium supplementation (%)	
	Y	N	Y	N	Y	N	Y	N	Y	N	Y	N	Y	N	Y	N
0 to 1	100 (17)	0.0 (0)	70 (7)	30 (3)	100 (7)	0.0 (0)	29.4 (5)	70.6 (12)	100 (1)	0.0 (0)	100 (17)	0.0 (0)	88.2 (15)	11.8 (2)	94.1 (16)	5.9 (1)
1 to 2	100 (33)	0.0 (0)	95.7 (22)	4.4 (1)	100 (10)	0.0 (0)	40.5 (15)	59.5 (22)	75 (3)	25 (1)	94.6 (35)	5.4 (2)	81.1 (30)	18.9 (7)	80.6 (29)	19.4 (7)
2 to 3	100 (41)	0.0 (0)	91.3 (21)	8.7 (2)	83.3 (15)	16.7 (3)	44.9 (22)	55.1 (27)	57.1 (4)	42.9 (3)	95.9 (47)	4.1 (2)	87.8 (43)	12.2 (6)	91.7 (44)	8.3 (4)
3 to 4	100 (43)	0.0 (0)	95.8 (23)	4.2 (1)	89.5 (17)	10.5 (2)	47.8 (22)	52.2 (24)	25 (1)	75 (3)	97.8 (45)	2.2 (1)	95.7 (44)	4.4 (2)	100 (41)	0.0 (0)
4 to 5	100 (19)	0.0 (0)	93.8 (15)	6.3 (1)	100 (5)	0.0 (0)	57.1 (12)	42.9 (9)	50 (1)	50 (1)	95.2 (20)	4.8 (1)	90.5 (19)	9.5 (2)	90.5 (19)	9.5 (2)
5 to 6	100 (4)	0.0 (0)	100 (3)	0.0 (0)	100 (2)	0.0 (0)	50 (3)	50 (3)	100 (1)	0.0 (0)	100 (6)	0.0 (0)	83.3 (5)	16.7 (1)	100 (5)	0.0 (0)
6 to 7	100 (6)	0.0 (0)	83.3 (5)	16.7 (1)	0.0 (0)	0.0 (0)	66.7 (4)	33.3 (2)	0.0 (0)	0.0 (0)	83.3 (5)	16.7 (1)	83.3 (5)	16.7 (1)	83.3 (5)	16.7 (1)

7 to 8	100 (6)	0.0 (0)	100 (3)	0.0 (0)	100 (3)	0.0 (0)	62.5 (5)	37.5 (3)	100 (2)	0.0 (0)	100 (8)	0.0 (0)	100 (8)	0.0 (0)	100 (8)	0.0 (0)
8 to 9	100 (2)	0.0 (0)	100 (1)	0.0 (0)	0.0 (0)	100 (1)	50 (1)	50 (1)	0.0 (0)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (1)	0.0 (0)
9 to 10	100 (2)	0.0 (0)	100 (2)	0.0 (0)	0.0 (0)	0.0 (0)	100 (2)	0.0 (0)	0.0 (0)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)
10 higher	100 (3)	0.0 (0)	100 (3)	0.0 (0)	0.0 (0)	0.0 (0)	66.7 (2)	33.3 (1)	0.0 (0)	0.0 (0)	100 (3)	0.0 (0)	100 (3)	0.0 (0)	100 (2)	0.0 (0)
Not found	100 (24)	0.0 (0)	78.6 (11)	21.4 (3)	80 (8)	20 (2)	52 (13)	48 (2)	100 (1)	0.0 (0)	88 (22)	12 (3)	76 (19)	24 (6)	80 (20)	20 (5)
Total lower then 5	100 (153	0 (0)	91.7 (88)	8.3 (8)	91.5 (54)	8.5 (5)	44.7 (76)	55.3 (4)	55.6 (10)	44.4 (8)	96.5 (164)	3.5 (6)	88.8 (151)	11.2 (19)	91.4 (149)	8.6 (14)
Total higher then 5	100 (23)	0 (0)	94.4 (17)	5.6 (1)	83.3 (5)	16.7 (1)	63 (17)	37 (10)	100 (3)	0 (0)	96.3 (26)	3.7 (1)	95.6 (25)	7.4 (2)	95.8 (23)	4.2 (1)

Table 9. Relation of the impact factor of the journals in the years of publications articles with the presence of some variables of interest.

Discussion

Gronthos and collaborators in 2000 innovated the field by isolating for the fist time stem cell from the dental pulp of permanent teeth, and therefore provided the basis for DPSCs isolation. Over the past 15 years many studies have been conducted using DPSCs. The promising results obtained, indicate that more efforts will be made for the use of these cells towards clinical applications and basics biology development. However this is the first systematic review regarding the isolation of DPSCs, and based on the frequency in each step of DPSCs isolation we developed a standardized protocol (Figure 2). However it is important to highlight that this protocol is based on frequency analysis and not in effectiveness of DPSCs isolation.

Within the years, the isolation of DPSCs has evolved, and currently, DPSCs can be isolated using two main techniques: explant (Kerkis, Kerkis et al. 2006, Karamzadeh, Eslaminejad et al. 2012, Lizier, Kerkis et al. 2012) and enzymatic (Kerkis, Kerkis et al. 2006, Karamzadeh, Eslaminejad et al. 2012). Moreover, the association between the enzymatic technique and the use of mechanical devices to intensify cell dissociation and the extracellular matrix, has also been used (Patil, Kumar et al. 2014).

Our findings revealed that 56.3% of all included studies chose the enzymatic technique, and 33.5% associated this methodology with the mechanical cell dissociation step. The enzymatic technique consists of removing DPSCs from the pulp by using enzymes, such as collagenase, dispase (Kerkis, Kerkis et al. 2006) and trypsin (Asgary, Nazarian et al. 2014), to dissociate cells from each other and from its extracellular matrix, in order to obtain an heterogeneous singularized cell suspension. However this process can inevitably damage the cell or induce its death (Huang, Sonoyama et al. 2006). In this regard, all articles described the enzyme used for DPSCs dissociation. Within the 222 studies included, only five families of enzymes were mentioned: collagenase, dispase, trypsin, thermolysin and DNAase. Considering these families of enzymes, different subtypes could be found, and therefore, 20 different combinations were noted in the articles. The most mentioned combination of enzymes (54.4% of total studies) was collagenase type I/dispase, while the second most common was collagenase type I alone (17.2%). It was also observed that in some cases there was no specification to the subtype of enzyme used, such as the association between collagenase and dispase, collagenase and trypsin and collagenase alone, representing respectively 6.9%, 2.5% and 4.4% of

total studies using the enzymatic technique. Since the dental pulp is a connective tissue and its extracellular matrix contains collagen as a major component (Lukinmaa and Waltimo 1992), the high percentage of authors that used collagenase and dispase in the enzymatic technique, two proteolytic enzymes that degraded collagen, is highly comprehensive (Koga, Muneta et al. 2008) (Rodbell 1964).

Regarding the concentration of enzymes, the group of authors that used collagenase type I in association with dispase, showed that there is no standardized quantity of each enzyme to promote cell disaggregation. Indeed, up to eight different concentrations of collagenase type I/dispase were mentioned. This high variability in concentrations is also observed with collagenase, collagenase type I and collagenase/dispase (Table 4). This lack of standardization in the enzyme and concentration could compromise DPSCs survival and function and hamper the initiation of stem cell isolation for various laboratories. Based on the results obtained from this systematic review we propose the use of collagenase type I/dispase in the concentration of 3 mg/mL and 4 mg/mL respectively.

In a sequence of temporal events, it is common that after enzymatic disaggregation, the dissociated pulp tissue is filtered through a falcon cell strain (Pisciotta, Carnevale et al. 2015). We observed that the filters pores used were 40, 70 and 100 μm, (10.4%, 69.8%, and 10.4% respectively, in relation to the total of 106 studies that used cell filtration). This process of filtration is used in order to remove any cell clump (Francis, Sachs et al. 2010), and therefore can be adapted according to the cell type used. For DPSCs, however, this systematic review suggests the use of 70 μm filters.

The explant technique, based on the outgrowth of cell from small tissue fragments, as well as the enzymatic technique, is able to isolate cells with the capacity to differentiate in multiple lineages (Hilkens, Gervois et al. 2013). This technique is cheaper than the enzymatic technique (Huang, Shagramanova et al. 2006) and allows the isolation of a relatively pure (not homogeneous) population of pulp stem cells (Kerkis, Kerkis et al. 2006). However only 9.9% of the authors chose to use this technique. It is speculated that this small number is related to the long time needed to conduct this method, since cells need up to 1-2 weeks to migrate from pulp tissue. Lizier and collaborators developed a cell culture method that preserves DPSCs viability for longer periods, minimizing DPSCs slow rate proliferation (Lizier, Kerkis et al. 2012). This methodology is based on the explant technique and consists in transferring tissue fragments to a different plate every time the desired cell density is

achieved. It allows the *de novo* cell migration to a new plate, enabling the achievement of large amount of pulp stem cells (Lizier, Kerkis et al. 2012).

A particular aspect of the explant technique is the size of tissue fragments. According to our data fragment dimension are not standardized. It was observed that 45.5% of the articles did not bring this information; 18.2% mentioned that tissue fragments were small, however did not specify the dimension used; 13.6% cite a single dimension; 18.2% two dimension and only 4.6% the three dimension used. The literature seems scarce in this sense and little is discussed about the relevance of the explant size in order to obtain success in isolating dental pulp stem cells with this technique technique. Recently, a study compared explants of bone tissue with two size fragments: up to 2 mm and 2 to 5 mm. It was observed that the size of the explants is not a factor capable of influencing osteoblast outgrowth (Verdugo, Saez-Roson et al. 2011), though this observation should be viewed with caution, since cell from different sources could behave differently. One important aspect when considering the size of the explant fragments is that it should not be excessively small at a point that it can compromise the explant attachment to the bottom of the culture plate, unfeasible the cell outgrowth. And on the other hand it should not be excessively large in order to compromise the diffusion of metabolites a nutrients between the surrounding medium and the explant, as well as dificulting the cell outgrowth.

Subsequently, after establishing the type of technique used for DSPCs isolation, it is necessary to establish the adequate culture medium and its supplements, which will enable the culture, *in vitro*, of the isolated cells (Pal, Hanwate et al. 2009). Our data showed that nearly 95.0% of the articles described the culture medium used in DPSCs cultivation in vitro (Table 1), indicating a very low risk of bias (Figure 1) and therefore highlighting its relevance. A total of 212 articles cited the medium used for DPSCs cultivation, with a variability of 19 different types. Of these, α-MEM, DMEM and DMEM/F12 were the most recommended types, representing respectively 52.3%, 21.1% and 4.1% of the total items included (Table 6). Pat et al. reported that α-MEM and DMEM/F12, can be used to isolate and successfully expand DPSCs and other mesenchymal stem cells (Pal, Hanwate et al. 2009). Recently the rate proliferation and the gene expression pattern of pulp stem cells from deciduous teeth was evaluated when α-MEM and DMEM/F12. It was observed that α-MEM and DMEM/F12 were the most suitable medium for dental pulp stem cell from deciduous

teeth isolation and long-term expansion (Lizier, Kerkis et al. 2012). The low-glucose DMEM was used in 3.2% of the articles evaluated, however the literature has not been shown effective for isolation, but efficient to support long-term expansion of dental pulp stem cell culture after cryopreservation (Lizier, Kerkis et al. 2012).

It is well known that the culture medium is not placed in contact with the cells alone, a series of components help to create an environment suitable for maintaining cell viability *in vitro*, aiming to re-create, as an ultimate goal, the stem cell niche where these cells maintain there undifferentiating state (Schofield 1978).

Supplementation components for cell culture medium are present in 90.0% of the included studies. Specifically in relation to serum 87.8% of the articles cited its use. Schukanen et al. aimed to approach DPSCs behavior when exposed *in vitro* to the cell culture medium supplemented with different concentrations of fetal calf serum (FCS) (2% and 10%) and association between insulin, transferrin and sodium selenite (ITS). The result shows that the ITS supplement promoted increased of DPSCs proliferative activity, reducing the time of cell duplication and not altering the cellular phenotype. It was also found that reducing the FCS volume of 10% to 2% the properties of DPSCs was compromised (diameter and viability cellular) and it was suggested that as the serum concentration can be reduced to 2% but the medium should be enriched by ITS and growth factor supplementation (Suchanek, Soukup et al. 2009). From the 222 articles evaluated, the ITS association was not identified, and If we observe in an isolated or in combination way with other supplements, insulin and transferrin, each one represent just 1.2% of the total. Sodium selenite is not mentioned as a supplement for any author. The supplements that were reported more often were two antibiotics: penicillin, present in 26.0% of studies and streptomycin 25.0%. Another supplementation component cited was L-ascorbic acid, present in 6.6% of all studies. However, the literature suggests that the use of 100 µM of ascorbic acid, 10% of FCS as supplementation for α-MEM medium culture it provides a means of cultivation considered ideal for cell DPSCs growth (Suchanek, Soukup et al. 2009).

Regarding the particularities towards serum supplement, we can analyze that the serum of animal origin is the most recommended in the literature, representing 98.0% of the total. The fetal bovine serum (FBS) and the FCS represent were the most cited ones in this review, respectively, 74.4% and 23.1%. It is believed that this may be due to the fact that its use for DPSCs cultivation is already widespread in the

literature (Gronthos, Mankani et al. 2000, Abdullah, Abdullah et al. 2014). However, serum from animal origin can present disadvantages such as the risk of infections and immunological reactions. Thus, it motivated the Ferro's group to investigate a replacement element. Thus, the literature has investigated human serum, present in 2.0% off all studies, at low concentrations as a valid substitute in medium with high concentration of FBS and therefore be useful for clinical applications of adult stem cells (Ferro, Spelat et al. 2012). Moreover, the human serum has shown to induce high rate of cell proliferation and enhances the osteogenic differentiation of DPSCs *in vitro* (Bressan, Ferroni et al. 2012). However, more studies are needed addressing different aspects of the impact of human serum in cell behavior, considering that this serum has not been frequently used, corresponding for 2.0% of all studies.

Having been checked the risk of bias of several variables of interest as well as the impact factor of the journals, in the year when the studies were published, in association will these variables, was possible to realize an analysis of the quality of the included studies. Succinctly, is highlighted that studies showed, in general, low risk of bias for most variables of interest. Nevertheless, given the high variability of different factors impact, the data collected suggests that the presence of description of the variables is founded on a relationship of dependency the relevance of variable and not determined by the impact factor of the journals.

Based on the frequency in each step of DPSCs isolation obtained in our systematic review we developed a standardized protocol (Figure 2). However it is important to highlight that this protocol is based on frequency analysis and not in effectiveness of DPSCs isolation.

Conclusion

In conclusion over the past 15 years many studies have been conducted using DPSCs, however this is the first systematic review regarding the isolation of DPSCs. The isolation of DPSCs showed great variability, hampering the development of standard protocols to achieve DPSCs *in vitro* with similar characteristics from those *in vivo*, and possibly influencing the results of the same evaluated outcomes. Finally, based in the results from the systematic review we proposed a standard protocol for DPSC isolation.

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Supplementary material

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Abdullah 2014	10-40 y			collagenase type I (Worthing Biochem, USA)	3 mg/mL	Overnight	70 µm strainer (BD FalconTM, USA)		α- MEM (BioWhittaker ™, USA)	FBS (Gibco™, Invitrogen, USA)	20%
										L-ascorbic acid 2-phosphate (Stem Cell Technologies, Canada)	100 µM
										L-glutamine (Gibco™, Invitrogen, Japan)	2 mM
										penicillin	100 U/mL
										streptomycin	100 µg/mL
Abu Kasim 2012	24–35 y			collagenase type I (Gibco, Grand Island, NY, USA)	3 mg/mL	40 min		Minced in small framents	0.5% DMEM-KO	penicillin (Invitrogen)	10000 µg/mL
										streptomycin (Invitrogen)	10000 µg/mL
										Glutamax (Invitrogen)	1 % 1 X
										FBS	10%
Agha-Hosseini 2010	18–28 y			collagenase (Sigma C-5138, St.Louis, MO, USA)				Fragmented in small pieces			
Ahmed 2011	20-29 y			collagenase type II (Worthington biochemical corporation, Lakwood, USA)	0.2%	30 min		Dissected into small pieces	EBM2 (Lonza, USA)	FBS	10%
										rhIGF-1 (Lonza, USA)	
										rhEGF (Lonza, USA)	
Akkouch 2014	18–25 y			collagenase type I (Invitrogen, Life Technologies, Burlington, CN, Canada)	3 mg/mL	60 min			α- MEM (Invitrogen Life Technologies)	Inactivated FCS (FBS, NCS, fetal clone II; Hyclone, Logan, UT)	10%
										penicillin (Schering, Pointe-Claire, QC, Canada)	200 U/mL
										streptomicin (Schering, Pointe-Claire, QC, Canada)	200 µg/mL
										amphotericin B (Sigma-Aldrich)	2.5 µg/mL
Al-Habib 2013	16–24 y			collagenase type I (Sigma Aldrich)	3 mg/mL	30-60 min	70 µm (Falcon, BD Labware)	Minced	α- MEM (Gibco Invitrogen)	SFB (Gemini, Bio-Products, Inc.)	10%
										L-glutamine (Gemini Bio-Products, Inc.)	2 mM
										L-ascorbic acid-2-phospate (Gemini Bio-Products, Inc.)	100 µM
				dispase (Sigma Aldrich)	4 mg/mL					penicillin-G (Gemini Bio-Products, Inc.)	100 U/mL
										streptomycin (Gemini Bio-Products, Inc.)	100 µg/mL
										fungizone (Gemini Bio-Products, Inc.)	0.25 µg/mL
Alongi 2010	14-22 y			collagenase type I	3 mg/mL	30-60 min	70 µm cell strainer	Minced	α- MEM	FBS	15%
				dispase	4 mg/mL					L-glutamine	2 mM
										L-ascorbic acid-2-phosphate	100 µM
Armiñán 2009	18-21 y			collagenase type A (Gibco, Grand Islands, NY)	2 mg/mL	90 min		Minced in small fragments (<1 mm 3)	Low glucose DMEM	FCS (Invitrogen)	10%
										antibiotics	

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Arthur 2008	19-35 y			collagenase type I	3 mg/mL	60 min	70 µm strainer		α- MEM	FCS	20%
										L-ascorbic acid 2-phosphate	100 µM
				dispase	4 mg/mL					L-glutamine	2 mM
										penicillin	100 U/mL
										streptomycin	100 µg/mL
Asgary 2014				collagenase	0.1%	20 min			DMEM	FBS (Invitrogen, Carlsbad, CA)	15%
				trypsin	0.05%					penicillin (Invitrogen, Carlsbad, CA)	100 U/mL
				EDTA	0.5 mmol/L					Fungizone (Invitrogen, Carlsbad, CA)	0.25 g/mL
				PBS	0.1 mol/L					streptomycin (Invitrogen, Carlsbad, CA)	100 U/mL
Atari 2011	14-60 y	Male		collagenase type I (Sigma)	3 mg/mL	60 min		Insulin syringe	Low glucose DMEM (60%) (Sigma) and MCDB-201 (40%) (Sigma)	ITS (Sigma)	1 X
										LA-BSA (Sigma)	1 X
										dexamethasone (Sigma)	10^{-9} M
										ascorbic acid 2-phosphate (Sigma)	10^{-4}M
										penicillin	100 U
										streptomycin (PAA)	1000 U
										FBS (Sigma)	2%
		Female								hPDGFBB (R&D Systems)	10 ng/mL
										EGF (R&D Systems)	10 ng/mL
										hLIF (Chemicon)	1000 U/mL
										Chemically Defined Lipid Concentrate (Gibco)	
										BSA (Sigma)	0.8 mg/mL
										ß-ME (Sigma)	55 µM
Atari 2012 (a)	18–27 y	Male		collagenase type I (Sigma)	3 mg/mL	60 min		Insulin syringe	Low glucose DMEM (60%) and MCDB-201 (40%) or DMEM (Biochrom)	ITS (Sigma)	1 X
										LA-BSA (Sigma)	1 X
										dexamethasone (Sigma)	10^{-9}M
										ascorbic acid 2-phosphate (Sigma)	10^{-4}M
										penicillin	100 U
		Female								streptomycin (PAA)	1000 U
										FBS (Sigma)	2%
										hPDGFBB (R&D Systems)	10 ng/mL
										EGF (R&D Systems)	10 ng/mL
										bFGF	2 ng/mL
										FBS (Hyclone)	10%

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Quantity
				Types	Concentration	Time	Filtering				
Atari 2012 (b)	14–60 y	Male		collagenase type I (Sigma)	3 mg/mL	60 min		Insulin syringe	Low glucose DMEM (60%) (Sigma) and MCDB-201 (40%) (Sigma)	ITS	1 X
										LA-BSA (Sigma)	1 X
										dexamethasone (Sigma)	10^{-9}M
										ascorbic acid 2-phosphate (Sigma)	10^{-4}M
										penicillin	100 U
										streptomycin (PAA)	1000 U
										FBS (Sigma)	2%
										hPDGFBB (R&D Systems)	10 ng/mL
		Female		dipase (Sigma)	4 mg/mL					EGF (R&D Systems)	10 ng/mL
										hLIF (Chemicon)	1000 U/mL
										Chemically Defined Lipid Concentrate (Gibco)	
										BSA (Sigma)	0.8 mg/mL
										ß-ME (Sigma)	55 µM
									DMEM (Biochrom)	bFGF	2 ng/mL
										FBS (Hyclone)	10%
Attar 2014				collagenase type I (Sigma, St. Louis, MO, USA)	3 mg/mL	60 min	70 µm cell strainer (BD Biosciences, San Jose, CA, USA)	Minced			
				dispase type II (Sigma, St. Louis, MO, USA)	4 mg/mL						
Bakopoulou 2011	16-18 y			collagenase type I (Gibco/BRL, Karlsruhe, Germany)	3 mg/mL	60 min	70 µm cell strainer (BD Biosciences, Heidelberg Germany)		α- MEM (Gibco/Invitrogen, Karlsruhe, Germany)	FBS (Lonza, Verviers, Belgium)	15%
										L-ascorbic acid phosphate (Biochrom AG, Berlin, Germany)	100 mM
										L-glutamine (Biochrom AG, Berlin, Germany)	2 mM
				dispase (Roche Diagnostics GmbH, Mannheim, Germany)	4 mg/mL					penicillin (Biochrom AG, Berlin, Germany)	100 U/mL
										streptomycin (Biochrom AG, Berlin, Germany)	100 mg/mL
										amphotericin B (Promo Cell, Heidelberg, Germany)	0.25 mg/mL
Batouli 2003	19-29 y			collagenase type I (Worthington Biochem, Freehold, NJ, USA)	3 mg/mL	60 min			α- MEM (Gibco BRL, Grand Island, NY, USA)	FCS (Equitech-Bio Inc., Kerrville, TX, USA)	15%
										L-ascorbic acid 2-phosphate (WAKO, Tokyo, Japan)	100 µM
				dispase (Boehringer Mannheim GmbH, Mannheim, Germany)	4 mg/mL					Lglutamine (Biofluids Inc., Rockville, MD, USA)	2 mM
										penicillin (Biofluids Inc., Rockville, MD, USA)	100 U/mL
										streptomycin (Biofluids Inc., Rockville, MD, USA)	100 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Bonnamain 2013	15–20 y	Male		collagenase type IA (Sigma)	3 mg/mL	60 min	70 µm cell strainer (Falcon)	Minced into small pieces	DMEM/Ham'sF12 (1/1,v/v)	glucose (Lonza, Basel,Switzerland)	33 mM
										HEPES (Lonza, Basel,Switzerland)	5 mM
		Female								streptomycin (Lonza, Basel,Switzerland)	5 µg/mL
				dispase (Gibco)	4 mg/mL					penicillin (Lonza, Basel,Switzerland)	5 UI/mL
										heat-inactivated FCS (Lonza, Basel,Switzerland)	10%
Bressan 2012	16-66 y			penicillin	100 U/mL	60 min	70 mm Falcon strainers (Becton & Dickinson, Franklin Lakes, NJ)		NH stem cell expansion (Miltenyi Biotec, Bergish Gladbach, Germany)		
				streptomycin	100 mg/mL						
				clarithromycin	0.6 mL of 500 mg/mL						
				collagenase type I	3 mg/mL						
				dispase	4 mg/mL						
				PBS	4 mL of 1 mol						
Cai 2011	18-28 y			collagenase type II (Sigma-Aldrich, St. Louis, MO, USA)	0.075%	30 min	Filtered (no described)		DMEM/F12 (1 : 1)	FBS	10%
				PBS						penicillin	100 U/mL
										streptomycin	100 µg/mL
Carinci 2008				penicillin	100 U/mL	60 min	70 µm cell strainers (Falcon; Becton & Dickinson, Sunnyvale, CA)		α- MEM	FBS (Invitrogen SRL, San Giuliano Milanese, Milan, Italy)	22.5%
				streptomycin	100 µg/mL					2P-ascorbic acid (Invitrogen SRL, San Giuliano Milanese, Milan, Italy)	100 µmol/L
				PBS	4 mL					L-glutamine (Invitrogen SRL, San Giuliano Milanese, Milan, Italy)	2 mmol/L
				clarithromycin	500 µg/mL					penicillin (Invitrogen SRL, San Giuliano Milanese, Milan, Italy)	100 U/mL
				collagenase type I	3 mg/mL					streptomycin (Invitrogen SRL, San Giuliano Milanese, Milan, Italy)	100 µg/mL
				dispase	4 mg/mL						
Carvalho 2012				trypsin				Cut	α- MEM	FBS (Gibco, UK)	10%
										penicillin (Gibco, UK)	1% (100 IU/mL)
										streptomycin (Gibco, UK)	1% (2.5 µg/mL)
				collagenase						amphotericin B (Gibco, UK)	2.5 µg/mL
										ascorbic acid (Sigma, USA)	50 µg/mL
Chen 2013	Mean of 26.5 y			collagenase type I (Invitrogen, Carlsbad, CA, USA)	3 mg/mL	60 min	40 µm cell strainer (Falcon BD, Franklin Lakes, NJ, USA)		α- MEM (Hyclone, Logan, UT, USA)	FBS (Hyclone)	20%
										L-ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA)	100 µM
	Mean of 23.4 y			dispase (Invitrogen)	4 mg/mL					penicillin (Sigma)	100 U/mL
										streptomycin (Invitrogen)	100 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Chen 2012	19-23 y			collagenase type I (Sigma Aldrich St. Louis, MO, USA)	3 mg/mL	30-60 min	70 µm cell strainer	Minced	α- MEM	FBS (Hyclone)	20%
										streptomycin (Roche)	100 mg/mL
				dispase (Sigma Aldrich St. Louis, MO, USA)	4 mg/mL					penicillin-G (Roche, Basel, Switzerland)	100 U/mL
Chen 2011	Mean of 25.5 y, 6–74 y	Male		collagenase type I (Invitrogen, Carlsbad, CA, USA)	3 mg / mL	60 min	40 µm cell strainer (Falcon BD, Franklin Lakes, NJ, USA)		α- MEM (Hyclone, Logan, UT, USA)	FBS (Hyclone)	20%
	Mean of 26.5 y, 6-74 y									penicillin (Sigma)	100 U/mL
	Mean of 23.4 y, 6-49 y	Female		dispase (Invitrogen)	4 mg / mL					streptomycin (Invitrogen)	100 µg/mL
	Mean of 27.7 y, 8-52 y									L-ascorbic acid-2-phosphate (Sigma, St. Louis, MO, USA)	100 µM
Choi 2012				collagenase type I (Gibco)	1 mg/mL	60 min			MEM	FBS (Hyclone)	10%
				dispase (Gibco)	2.4 mg/mL					AA (Gibco)	1%
Chun 2011 (a)				collagenase type I (Worthington, Lakewood, NJ)	3 mg/mL		100 µm nylon mesh		α- MEM (Invitrogen)	FBS (Invitrogen)	10%
				dispase (Invitrogen, Grand Island, NY)	4 mg/mL						
Chun 2011 (b)				collagenase type I (Worthington)	3 mg/mL	30 min	100 µm nylon mesh	Chopped in small pieces	α- MEM (Invitrogen)	FBS (Invitrogen)	10%
										penicillin	100 units/mL
				dispase (Invitrogen)	4 mg/mL					streptomycin	100 µg/mL
Cmielova 2013	Mean of 19 y, 12-23 y	Male		collagenase (Sevapharma, Prague, Czech Republic)		70 min			α- MEM (Invitrogen, Carlsbad, CA, USA)	FCS (PAA, Dartmouth, MA, USA)	2%
										EGF (PeproTech, Rocky Hill, NJ, USA),	10 ng/mL
										PDGF (PeproTech, Rocky Hill, NJ, USA)	10 ng/mL
										ascorbic acid (Sigma, St Louis, MO, USA)	
										glutamine (Invitrogen, Carlsbad, CA, USA)	2%
		Female		dispase (Invitrogen, Carlsbad, CA, USA)						streptomycin (Invitrogen, Carlsbad, CA, USA)	
										penicillin (Invitrogen, Carlsbad, CA, USA)	
										gentamycin (Invitrogen, Carlsbad, CA, USA)	
										dexamethasone (Sigma, St Louis, MO, USA)	

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Collart-Dutilleul 2014	15–18 y			collagenase type I (BD Biosciences, Bedford, MA)	3 mg/mL	60 min	70 µm Falcon strainers		α-MEM	FBS (Invitrogen, Carlsbad, CA, USA)	10%
				dispase (BD Biosciences, Bedford, MA)	4 mg/mL					penicillin (Invitrogen, Carlsbad, CA, USA)	100 U/mL
										streptomycin (Invitrogen, Carlsbad, CA, USA)	100 µg/mL
Cui 2014				collagenase type I (Sigma Aldrich, St Louis, MO)	3 mg/mL	60 min	70 µm strainer (Carrigtwohill Co, Cork, Ireland)		DMEM	FBS (Gibco-BRL, Grand Island, NY)	15%
				dispase (Sigma Aldrich)	4 mg/mL					penicillin (Gibco-BRL, Grand Island, NY)	100 U/mL
										streptomycin (Gibco-BRL, Grand Island, NY)	100 mg/mL
Cui 2013				PBS		60 min	70 µm strainer (BD Falcon, San Jose, CA, USA)		DMEM	FBS (Gibco-BRL, Grand Island, NY)	15%
				collagenase type I (Sigma, St Louis, MO, USA)	3 mg/mL					penicillin (Gibco-BRL, Grand Island, NY, USA)	100 U/mL
										streptomycin (Gibco-BRL, Grand Island, NY)	100 U/mL
Dai 2012	Mean of 18+ -3.2, 16-25 y		0.5-1 mm						DMEM (Gibco)	FBS (Hyclone)	20%
										antiobiotics	
D'Alimonte 2013	Mean of 17 y	Male		MEM		60 min	70 µm strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA)		MEM	penicillin (Invitrogen, Milan, Italy)	100 U/mL
				penicillin (Invitrogen, Milan, Italy)	100 U/mL					streptomycin (Invitrogen, Milan, Italy)	100 µg/mL
				dispase (Roche, Monza, Italy)	4 mg/mL						
		Female		streptomycin (Invitrogen, Milan, Italy)	100 µg/mL					clarithromycin (Menarini, Florence, Italy)	500 µg/mL
				collagenase type I (Sigma)	3 mg/mL						
				clarithromycin (Menarini, Florence, Italy)	500 µg/mL						
D'Alimonte 2011	Mean of 17 y	Male		α-MEM (Sigma, Mylan, Italy)		60 min	70 µm strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA)		α-MEM (Sigma, Mylan, Italy)	penicillin (Invitrogen, Milan, Italy)	100 U/mL
				penicillin (Invitrogen, Milan, Italy)	100 U/mL					clarithromycin (Menarini, Florence, Italy)	500 µg/mL
				collagenase type I (Sigma)	3 mg/mL						
				streptomycin (Invitrogen, Milan, Italy)	100 µg/mL					streptomycin (Invitrogen, Milan, Italy)	100 µg/mL
		Female		dispase (Roche, Monza, Italy)	4 mg/mL						
				clarithromycin (Menarini, Florence, Italy)	500 µg/mL						
D'Aquino 2009		Male					70 µm strainer	Realized (not described)	MEM (Cambrex, Charles City, IA, USA)	FBS (Invitrogen, San Giuliano Milanese, Italy)	20%
		Female									
D'Aquino 2007		Male		collagenase type I	3 mg/mL		70 µm strainer (Falcon, Becton Dickinson, Franklin Lakes, NJ, USA)		MegaCell (Sigma, Milan, Italy)	FBS (Invitrogen, San Giuliano Milanese, Italy)	10%
				dispase	4 mg/mL					2P-ascorbic acid (Invitrogen, San Giuliano Milanese, Italy)	100 µM
				PBS	1 mol					L-glutamine (Invitrogen, San Giuliano Milanese, Italy)	2 mM
		Female		penicillin	100 U/mL					penicillin (Invitrogen, San Giuliano Milanese, Italy)	100 U/mL
				clarithromycin	0.6 mL (500 µg/mL)					streptomycin (Invitrogen, San Giuliano Milanese, Italy)	100 µg/mL
				streptomycin	100 µg/mL						

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
de Rosa 2011	21-45 y			collagenase type I	3 mg/mL	60 min			α-MEM	FBS (Invitrogen)	20%
										2P-ascorbic acid (Invitrogen)	100 µM
				dispase	4 mg/mL					L-glutamine (Invitrogen)	2 mM
										penicillin (Invitrogen)	100U/mL
				PBS						streptomycin (Invitrogen)	100 µg/mL
de Souza 2010	9-15 y		Realized (not described)	colagenase type I (Gibco™)	3 mg/mL	60 min			DMEM (Gibco™)	FBS (Gibco™)	20%
										streptomycin (Gibco™)	100 mg/mL
				dispase (Gibco™)	4 mg/mL						
										penicillin (Gibco™)	100 units/mL
Demircan 2011	20 y	Male		collagenase type I (Sigma Aldrich, St Louis, MO USA)					MEM Earle's (FG0325; Biochrom, Holliston, MA, USA)	heat-inactivated FBS (Invtrogen/Gibco, Grand Island NY, USA)	15%
										penicillin (Invitrogen/Gibco)	100 IU/mL
										streptomycin (Invitrogen/Gibco)	100 mg/mL
Diomede 2013	18-25 y		Cut into small pieces						MSCM medium (Lonza Walkersville Inc., Walkersville, MD, USA)		
Dissanayaka 2012	18-25 y			collagenase type I (Gibco Invitrogen, Carlsbad, CA)	3 mg/mL	60 min	70 µm (BD Biosciences, Franklin Lakes, NJ)		α- MEM	FBS	15%
										L-ascorbic acid-2-phosphate	
										penicillin-G	100 U/mL
				dispase (Gibco Invitrogen)	4 mg/mL					streptomycin	100 µg/mL
										fungizone (Gemini Bio-Products, Woodland, CA)	0.25 µg/mL
Dissanayaka 2011				collagenase type I (Gibco Invitrogen, Carlsbad, CA)	3 mg/mL				α- MEM	FBS (Gemini Bio-Products, Woodland, CA)	15%
										L-ascorbic acid-2-phosphate (Gemini Bio-Products, Woodland, CA)	
				dispase (Gibco-Invitrogen)	4 mg/mL					penicillin-G (Gemini Bio-Products, Woodland, CA)	100 U/mL
										streptomycin (Gemini Bio-Products, Woodland, CA)	100 µg/mL
										fungizone (Gemini Bio-Products, Woodland, CA)	0.25 µg/mL
Djouad 2010	16-26 y			collagenase type I (Sigma)	3 mg/mL	30-60 min	70 µm cell strainer (Becton Dickinson)		α-MEM	FBS (Gemini Bio-Products)	20%
										L-glutamine (Gemini Bio-Products)	2 mmol/L
										L-ascorbic acid-2-phosphate (Gemini Bio-Products)	100 µmol/L
				dispase (Sigma)	4 mg/mL					penicillin-G (Gemini Bio-Products)	100 U/ml
										streptomycin (Gemini Bio-Products)	100 µg/ml
										fungizone (Gemini Bio-Products)	0.25 µg/ml

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Dolatshahi-Pirouz 2010	21 y			MEM (Gibco, Taastrup, Denmark)		30 min	100 µm strainer (BD Biosciences, Discovery Labware,Bedford, MA)		MEM (Gibco, Taastrup, Denmark)	FBS (PAA Laboratories, Linz, Austria)	10%
				dispase II (Roche Diagnostics Mannheim, Germany)	2.4 U/mL					penicillin (DuraScan Medical Products AS, Odense, Denmark)	25.000 IU/mL
				collagenase type I (Worthington Biochem, Freehold, NJ)	3 mg/mL					streptomycin (DuraScan Medical Products AS, Odense, Denmark)	25 mg/mL
Duailibi 2011				collagenase type II (Sigma-Aldrich St. Louis, MO, USA)	0.4 mg/mL	30–40 min	40 µm cell strainer	Minced into small pieces and less than 1 m in size in fresh HBBS			
				dispase I (Boehringer Mannheim, Indianapolis IN, USA)	0.2 mg/mL						
Ebrahimi 2011			Minced into small fragments in medium			30-60 min	70 µm cell strainer (BD Biosciences, Bedford, MA,USA)	Minced into small fragments in medium	α-MEM (Invitrogen, Carlsbad, CA, USA)	FBS (Invitrogen, USA)	20%
				collagenase (Roche, Mannheim, Germany)	1 mg/mL					penicillin	100 U/mL
				dispase (Roche, Mannhein, Germany)	3 mg/mL					streptomycin	100 µg/mL
										amphotericin B	25 ng/mL
Egbuniwe 2011				collagenase type I (Sigma Aldrich)	3 mg/mL	30 min		Minced into small pieces 1–2 mm by 3–4 mm	DMEM (Pharmakine)	FBS	10%
				dispase (Sigma Aldrich)	4 mg/mL					penicillin	2.5 x 1.000 U/mL
										streptomycin	2.5 mg/mL
Eleutério 2013	20-35 y		Small pieces						MSCM medium (Lonza Verviers Company, Belgium)		

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Eslaminejad 2013	20-25 y	Male		collagenase type I (Sigma, St Louis, MO, USA)	3 mg/mL	30 min					
				dispase (Sigma, St Louis, MO, USA)	4 mg/mL						
				FBS (Gibco)	15%						
				DMEM	3 mL						
Eslaminejad 2010 (a)	20-25 y			collagenase type I (Sigma, Germany)	3 mg/mL	30 min			DMEM (Sigma, Germany)	FBS (Sigma, Germany)	15%
				dispase (Sigma, Germany)	4 mg/mL					penicillin (Sigma, Germany)	100 IU/mL
				DMEM (Gibco, Germany)	3 mL					streptomycin (Sigma, Germany)	100 µg/mL
				FBS (Gibco, Germany)	15%						
Eslaminejad 2010 (b)	20-25 y			collagenase type I (Sigma, Germany)	3 mg/mL	45 min					
				dispase (Sigma, Germany)	4 mg/mL						
				DMEM (Gibco, Germany)	3 mL						
				FBS (Gibco, Germany)	15%						
Eslaminejad 2009	20-25 y			collagenase type I (Sigma, Germany)	3 mg/mL	30 min					
				dispase (Sigma, Germany)	4 mg/mL						
				DMEM (Gibco, Germany)	3 mL						
				FBS (Gibco, Germany)	15%						
Eubanks 2014	15–22 y			collagenase		60 min			α-MEM	FBS	15%
										HS	
				dispase II						HS (FN coating on the bottom of the flask)	
Fang 2013	16-30 y			Trypsin solution:	10 X	60 min	200 mesh/70 µ cell strainer	Cut into pieces with curved microscissors	α-MEM	FBS	15%
				collagenase type I	3 mg/mL					glutamine	2 mM
				dispase	4 mg/mL						

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Feng 2014	13-23 y			collagenase type I	3 mg/mL	60 min	70 µm cell strainer (BD Falcon)		DMEM	FBS	10%
										penicillin	100 U/mL
										streptomycin	100 mg/mL
Feng 2013 (a)	13-23 y			collagenase type I	3 mg/mL	60 min	70 µm cell strainer (BD Falcon)		DMEM	FBS	10%
										penicillin	100 U/mL
										streptomycin	100 mg/mL
Feng 2013 (b)	45-50 y			collagenase type I (Sigma, Germany)	3 mg/mL	60 min		Cut	DMEM	FBS (Gibco, USA)	10%
										penicillin (Gibco, USA)	100 U/mL
				dispase (Sigma Germany)	4 mg/mL					streptomycin (Gibco, USA)	100 U/mL
Foudah 2014									DMEM	FBS	10%
Gabanyi 2013				collagenase type IA (Sigma Aldrich, Brazil)	3 mg/mL				α-MEM (Sigma Aldrich, Brazil)	FCS (Cultilab, Brazil)	10%
										ciprofloxacin (Bayer, Brazil)	
Gandia 2008	18-21 y			collagenase type I (Gibco, Grand Island, NY)	2 mg/mL	90 min		Minced into small fragments	Low glucose DMEM	FCS (Invitrogen)	10%
										antibiotics	
Gay 2014				collagenase type I (Worthington Biochem, Freehold, NJ, USA)	3 mg/mL						
				dispase (Worthington Biochem, Freehold, NJ, USA)	4 mg/mL						
Giorgini 2011	18-20 y			collagenase type I (Gibco, Limited, Uxbridge, Unit, Kingdom)	3 mg/mL	60 min	70 µm BD Falcon strainer (Becton & Dickinson, Sunnyvale, CA, USA)		α-MEM	FBS (Gibco Limited, Uxbridge, United Kingdom)	10%
										penicillin-G (Gibco Limited, Uxbridge, United Kingdom)	100 U/mL
										streptomycin (Gibco Limited, Uxbridge, United Kingdom)	100 µg/mL
										fungizone (Gibco Limited, Uxbridge, United Kingdom)	0.25 µg/mL
Govindasamy 2011	24 – 35 y			collagenase type I (Gibco, Grand Island, NY, USA)	3 mg/mL	40 min		Minced into small fragments	DMEM-KO (Invitrogen, Carlsbad, CA, USA)	penicillin (Invitrogen)	0.5%
										streptomycin (Invitrogen)	10.000 µg/mL
										glutamax (Invitrogen)	1% 1X
										FBS	10%
Govindasamy 2010 (a)	24-35 y			collagenase type I (Gibco, Grand Island, NY)	3 mg/mL	40 min		Minced into small fragments	α-MEM (Invitrogen, Carlsbad, CA)	penicillin (Invitrogen)	0.5%
										streptomycin (Invitrogen)	10.000 µg/mL
										glutamax (Invitrogen)	1% 1X
										FBS	10%
Govindasamy 2010 (b)	14–25 y			collagenase type I (Gibco, Grand Island, NY, USA)	3 mg/mL	40 min		Minced into small fragments	DMEM-KO (Invitrogen, Carlsbad, CA)	FBS (Hyclone, Logan, UT)	10%
									DMEM-F12 (Invitrogen, Carlsbad, CA)	glutamax-1 (Invitrogen)	200 mM
									Low glucose DMEM (Invitrogen, Carlsbad, CA)	penicillin (Invitrogen)	
										streptomycin (Invitrogen)	

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Graziano 2008	21–45 y			penicillin	100 U/mL	60 min	70 µm Falcon strainers (Becton & Dickinson, Franklin Lakes, NJ)		α-MEM (Invitrogen, San Giuliano Milanese, Milan, Italy)	FCS (Invitrogen, San Giuliano Milanese, Milan, Italy)	20%
				streptomycin	100 µg/mL					2P-ascorbic acid (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 µM
				claritromycin	0.6 mL, 500 µg/mL					L-glutamine (Invitrogen, San Giuliano Milanese, Milan, Italy)	2 mM
				PBS	4 mL					penicillin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 U/mL
				collagenase type I	3 mg/mL					streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mg/mL
				dispase	4 mg/mL						
Graziano 2007	25-45 y			penicillin	100 U/mL	60 min	70 µm Falcon strainers (Becton & Dickinson, Franklin Lakes, NJ)		α-MEM (Invitrogen, San Giuliano Milanese, Milan, Italy)	FCS (Invitrogen, San Giuliano Milanese, Milan, Italy)	20%
				streptomycin	100 µg/mL					2P-ascorbic acid (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 µM
				claritromycin	0.6 mL, 500 µg/mL					L-glutamine (Invitrogen, San Giuliano Milanese, Milan, Italy)	2 mM
				PBS	4 mL					penicillin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 U/mL
				collagenase type I	3 mg/mL					streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mg/mL
				dispase	4 mg/mL						
Gronthos 2000	19-29 y			collagenase type I (Worthington Biochem, Freehold, NJ)	3 mg/mL	60 min	70 µm strainers (Falcon)		α-MEM (Gibco-BRL)	FCS (Equitech-Bio, Kerrville, TX)	20%
										L-ascorbic acid 2-phosphosphate (Wako Pure Chemicals Osaka)	100 µM
				dispase (Boehringer Mannheim)	4 mg/mL					L-glutamine (Biofluids, Rockville, MD)	2 mM
										penicillin (Biofluids, Rockville, MD)	100 U/mL
										streptomycin (Biofluids, Rockville, MD)	100 µM
Han 2010				collagenase type I (Sigma, St. Louis, MO, USA)	3 mg/mL	720 min		Minced	α-MEM (Sigma)		
				dispase (Sigma)	4 mg/mL						
				trypsin	3 mL of 0.25%	15 min					
Han 2008				collagenase type I (Sigma, St. Louis, MO, USA)	3 mg/mL	720 min		Minced	α-MEM (Sigma)		
				dispase (Sigma)	4 mg/mL						
				trypsin	3 mL of 0.25%	15 min					
Haveleck 2013				collagenase (Sevapharma, Prague, Czech Republic)		70 min			α-MEM (Invitrogen)	heat-inactivated FCS (PAA, Dartmouth, NH)	2%
										EGF (PeproTech, Rocky Hill,NJ)	10 ng/mL
										PDGF (PeproTech, Rocky Hill,NJ)	10 ng/mL
										L-ascorbic acid (Sigma-Aldrich, St. Louis, MO)	0.2 mM
										glutamine (Invitrogen)	2%
				dispase (Invitrogen, Carlsbad, CA)						penicillin (Invitrogen)	100 U/mL
										streptomycin (Invitrogen)	100 U/mL
										dexamethasone (Sigma)	50 nmol/L
										ITS (Sigma)	10 µl/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
He 2014	18-22 y			collagenase type I (Sigma, St. Louis, MO)	3 mg/mL	30 min		Minced	α-MEM (Invitrogen, Carlsbad, CA)	FBS (Gibco-BRL, Grand Island, NY)	
										penicillin-G	100 units/mL
				dispase (Sigma)	4 mg/mL					streptomycin (Invitrogen)	100 mg/mL
He 2013	18-22 y			collagenase type I (Sigma)	3 mg/mL	30-60 min	70 mm cell strainer (Becton/Dickinson, Franklin Lakes, NJ, USA)	Cut into small pieces	α-MEM (Invitrogen, Carlsbad, CA, USA)	glutamine (Invitrogen)	0.292 mg/mL
										penicillin G (Sigma)	100 units/mL
										streptomycin (Sigma)	100 mg/mL
				dispase (Sigma)	4 mg/mL					ascorbic acid (Sigma)	50 mg/mL
										FBS (Gibco-BRL., Life Technologies Inc, Gaithersburg, MD, USA)	
He 2008	19-29 y			collagenase type I	3 mg/mL	60 min	70 µm strainer (Falcon™, VWR International, USA)	Minced	α-MEM (Gibco-BRL, USA)	FCS (Gibco-BRL, USA)	20%
										L-glutamine (Gibco-BRL, USA)	2 mmol/L
				dispase	4 mg/mL					penicillin (Gibco-BRL, USA)	100 U/mL
										streptomycin (Gibco-BRL, USA)	100 U/mL
Hilkens 2013	15-20 y			collagenase type I (Sigma-Aldrich, St. Louis, Mo., USA)	3 mg/mL		70 µm cell strainer	Minced into fragments of 1–2 mm^3	α-MEM	L-glutamine	2 mM
				dispase (Sigma Aldrich, St. Louis, Mo., USA)	4 mg/mL					penicillin (Invitrogen, (Carlsbad, Calif., USA)	100 U/mL
			Minced into fragments of 1–2 mm^3							streptomycin (Invitriogen, Carlsbad, Calif., USA)	100 µg/ml
										FBS (Biochrom, Berlin Germany)	10%
Hirata 2010				collagenase type I (Walko Pure Chemicals, Osaka Japan)	3 mg/mL	60 min			DMEM (Invitrogen, Eugene, OR)	FBS (HyClone; Thermo-Scientific, South Logan, UT)	10%
										penicillin (Invitrogen)	100 U/mL
										streptomycin (Invitrogen)	100 µg/ml
										amphotericin (Invitrogen)	0.25 µg/mL
Hoss 2013				collagenase type I (Sigma, Steinheim, Germany)	3 mg/mL	60 min	70 µm strainer (BD, Germany)		α-MEM (Gibco, Karlsruhe, Germany)	FCS (PAN Biotech, Aidenbach, Germany)	20%
										L-ascorbic-acid-2-phosphate (Sigma)	100 µm
				dispase (Sigma, Steinhei, Germany)	4 mg/mL					Gentamycin (Gibco)	50 µg/mL
Huang 2010 (a)	14-22 y			collagenase type I (Sigma Aldrich , Sigma, St. Louis, MO)	3 mg/mL	30 min	70 mm strainer		DMEM (Kibbutz Beit Haemek, Israel)	FBS (Kibbutz Beit Haemek, Israel)	10%
										penicillin-Gm (Kibbutz Beit Haemek, Israel)	100 U/mL
				dispase (Sigma Aldrich, Sigma, St. Louis, MO)	4 mg/mL					streptomycin (Kibbutz Beit Haemek, Israel)	100 µg/ mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Huang 2010 (b)	16–24 y			collagenase type I (Sigma Aldrich, St. Louis, MO)	3 mg/mL	30–60 min	70 µm cell strainer (Falcon; BD Labware, Franklin Lakes, NJ)	Minced	α- MEM (Gibco Invitrogen, Carlsbad, CA)	FBS (Gemini Bio-Products, Woodland, CA)	15–20%
										L-glutamine (Gemini Bio-Products)	2 mM
										L-ascorbic acid-2-phospate (Gemini Bio-Products)	100 µM
				dispase (Sigma–Aldrich, St. Louis, MO)	4 mg/mL					penicillin-G (Gemini Bio-Products)	100U/mL
										streptomycin (Gemini Bio-Products)	100 mg/mL
										fungizone (GeminiBio-Products)	0.25 mg/mL
Huang 2009	41 y			collagenase type I (Invitrogen)	3 mg/mL	60 min	40 µm cell strainer (Falcon, BD, Franklin Lakes, NJ)		α-MEM (Hyclone, Logan, UT)	FBS (Hyclone)	20%
										L-ascorbic acid-2-phosphate (Sigma, St Louis, MO)	100 µmol/L
				dispase (Invitrogen)	4 mg/mL					penicillin (Invitrogen)	100 U/mL
										streptomycin (Invitrogen)	100 µg/mL
Huang 2008	20 y	Male		collagenase type I (Invitrogen)	3 mg/mL	60 min	40 µm cell strainer (Falcon, BD, Franklin Lakes, NJ, USA)		α- MEM (Hyclone, Logan, UT, USA)	FBS (Hyclone)	20%
										L-ascorbic acid-2-phosphate (Sigma, St Louis, MO, USA)	100 µM
				dispase (Invitrogen)	4 mg/mL					penicillin (Sigma)	100 units/mL
										streptomycin (Invitrogen)	100 µg / mL
Ishkitiev 2012				collagenase type I (Wako Pure Chemicals, Osaka, Japan)	3 mg/mL	60 min			DMEM (Invitrogen, Eugene, OR)	FCS (HyClone, Logan, UT),	10%
										penicillin (Meiji, Tokyo, Japan)	100 U/mL
										kanamycin (Meiji)	100 mg/mL
Ishkitiev 2010				collagenase type I (Wako Pure Chemicals, Osaka, Japan)	3 mg/mL	60 min			DMEM (Invitrogen, Eugene, OR)	FCS (HyClone, Logan, UT),	10%
										penicillin (Meiji, Tokyo, Japan)	100 U/mL
										kanamycin (Meiji)	100 mg/mL
Jeon 2011	16–18 y			collagenase type I	1 mg/mL	30 min	100 µm nylon cell strainer	Minced by using a sterilized scalpel	A-DMEM	FBS	10%
										penicillin	1%
										streptomycin	1%
Jin 2013	18-25 y			collagenase type I (Worthing, Biochem, Freehold, NJ)	3 mg/mL	60 min	40 µm strainer (Falcon, BD Labware, Franklin Lakes, NJ)		α- MEM (Gibco, BRL, Grand Island, NY)	FCS (Equitech-Bio, Inc., Kerrville, TX)	10%
										ascorbic acid 2-phosphate (WAKO, Tokyo, Japan)	100 µM
				dispase (Boehringer, Mannheim, Germany)	4 mg/mL					glutamine (Biofluids, Rockville,MD)	2 mM
										penicillin (Biofluids, Rockville,MD)	100 U/mL
										streptomycin (Biofluids, Rockville,MD)	100 µg/mL
Kadar 2009	18-26 y			collagenase type I (Sigma)	3 mg/mL	60 min	70 µm strainer (Falcon)		α- MEM (Gibco, BRL)	FBS (Gibco, BRL)	20%
										L-ascorbic acid 2-phosphate (Sigma)	100 µM
				dispase (Roche)	4 mg/mL					L-glutamine (Gibco, BRL)	2 mM
										penicillin (Gibco, BRL)	100 units/mL
										streptomycin (Gibco, BRL)	100 mg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Kanafi 2014	18-40 y			collagenase (Sigma Aldrich, St. Louis)	2 mg/mL	60 min		Minced into small pieces	DMEM-KO	FBS (Himedia, Mumbai, India)	10%
										L-glutamine (Himedia, Mumbai, India)	5 mM
										penicillin (Himedia, Mumbai, India)	50 U/mL
										streptomycin (Himedia, Mumbai, India)	50 U/mL
Kanafi 2013 (a)	5-40 y			collagenase	2 mg/mL	60 min		Minced into small pieces			
Kanafi 2013 (b)	5 - 40 y			collagenase	2 mg/mL	60 min		Minced	DMEM-KO (Invitrogen, Carlsbad, CA, USA)	FBS (Hyclone, Victoria, Australia)	10%
										L-glutamine (Invitrogen)	5 mM
										penicillin (Invitrogen)	50 U/mL
										streptomycin (Invitrogen)	50 U/mL
Kanafi 2013 (c)	5 - 40 y			collagenase	2 mg/mL	60 min		Minced into small pieces using		FBS (Hyclone, Victoria, Australia)	10%
										L-glutamine (Invitrogen)	5 mM
										penicillin (Invitrogen)	50 U/mL
										streptomycin (Invitrogen)	50 U/mL
Kanafi 2013 (d)	18-30 y			collagenase (Sigma Aldrich, St. Louis, MO, USA)	0.5 mg/mL				DMEM-KO (Gibco Grand Island, NY, USA)	FBS (Hyclone, Logan, UT, USA)	10%
										α-glutamine (Gibco)	200 mM/L
										penicillin (Gibco)	100 U/mL
										streptomycin (Gibco)	100 U/mL
Karaoz 2011	17-25 y			collagenase type I (Sigma Aldrich, St. Louis, MO, USA)	1%				MEM-Earle (Biochrom)	FBS (Invitrogen, Gibco, Grand Island, NY, USA)	15%
										penicillin (Invitrogen, Gibco)	100 IU/mL
										streptomycin (Invitrogen, Gibco)	100 µg/mL
Karbanová 2011	17-23 y			collagenase (Sevapharma, Prague, Czech Republic)		70 min	70 µm cell strainer (BD Biosciences, Heidelberg, Germany)	Realized (not described)	α-MEM (Gibco, Paisley, UK)	ascorbic acid 2-phosphate	0.2 mM
										dexamethasone	50 nM
										L-glutamine (Gibco, Paisley, UK)	2 mM
				dispase (Gibco)	2 mg/mL					penicillin (Gibco, Paisley, UK)	100 U/mL
										streptomycin (Gibco, Paisley, UK)	100 µg/mL
Karbanová 2010	17-23 y			collagenase (Sevapharma, Prague, Czech Republic)	0.2 mg/mL	70 min	70 µm cell strainer (BD Falcon)	Realized (not described)	α-MEM (Gibco)	dexamethasone (Sigma)	50 nM
										ascorbic acid 2-phosphate (Sigma)	0.2 mM
										L-glutamine (Gibco)	2 mM
										penicillin (Gibco)	100 U/mL
				dispase (Gibco, Paisley, UK)	2 mg/mL					streptomycin (Gibco)	100 µg/mL
										FCS (PAA Laboratories, Linz, Austria)	2%
										EGF (PeproTech, London, UK)	10 ng/mL
										PDGF-BB (PeproTech)	10 ng/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Kawanabe 2012	18-27 y			low-glucose DMEM (Invitrogen, Carlsbad, CA, USA)		60 min			low-glucose DMEM (Invitrogen,Carlsbad, CA, USA)	heat-inactivated FBS (Hyclone, Logan, UT, USA)	15%
				collagenase type II (Worthington, Lakewood, NJ, USA)	5 mg/mL					penicillin (Invitrogen)	100 U/mL
				dispase I (Roche, Penzberg, Germany)	2.5 mg/mL					streptomycin (Invitrogen)	100 µg/mL
Kellner 2014	12-30 y			collagenase type I (Invitrogen, Darmstadt, Germany)	4 mg/mL	60 min			α-MEM (Lonza Group AG, Basel, Switzerland)	FBS (Biotech GmbH, Aidenbach, Germany)	10%
				dispase (Invitrogen)	2 mg/mL					penicillin (Biochrom AG, Berlin, Germany)	100 U/mL
										streptomycin (Biochrom AG, Berlin, Germany)	100 µg/mL
Khanna-Jain 2012	Mean of 23 ± 2.5 years, 21–26 y			collagenase type I (Invitrogen)	3 mg/mL	60 min	100 µm cell strainer (Falcon; BD Labware, Franklin lakes, NJ, USA)	Minced by using scalpels	DMEM)/F-12 (1:1) (Gibco Life Technologies, Paisley, UK)	L-alanyl-l-glutamine (GlutaMAX I; Life Technologies)	1%
				dispase (Invitrogen, Paisley, Scotland, UK)	4 mg/mL					allogenic humam serum	20%
										AA	1%
Kim 2013				collagenase type I (Worthington, USA)	3 mg/mL	60 min	70 µm strainer (BD Falcon, USA)		α-MEM (Hyclone, South Logan,UT)	FBS (Hyclone)	10%
										AA (Gibco)	1%
Kim 2011	18 and 19 y	Male		collagenase type I (Gibco, Grand Island, NY, USA)	3 mg/mL	60 min	70 µm strainer (BD Biosciences, Franklin Lakes NJ, USA)		α-MEM (Gibco)	FBS (Gibco)	15%
										ascorbic acid 2-phospate (Fluka Chemie GmbH, Buchs, Switzerland)	100 mol/L
		Female		dispase (Gibco)	4 mg/mL					penicillin (Gibco)	100 U/mL
										streptomycin (Gibco)	100 µg/mL
Király 2011	19-35 y			collagenase type I	3 mg/mL	60 min	70 µm strainer	Minced	α-MEM	ascorbic acid 2-phosphate	100 µM
										L-glutamine	2 mM
										penicillin	100 U/mL
				dispase type II	4 mg/mL					streptomycin	100 µg/mL
										FCS	10%
Király 2009	19–55 y			collagenase type I	3 mg/mL	60 min	70 µm strainer	Minced	α-MEM	ascorbic acid 2-phosphate	100 µM
										L-glutamine	2 mM
										penicillin	100 U/mL
				dispase type II	4 mg/mL					streptomycin	100 µg/mL
										FCS	10%

Author and year	Age	Gender	Isolation techniques					Culture Medium	Medium supplementation	
			Explant	Enzymatic			Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering			
Kolind 2014				MEM (Gibco,Taastrup, Denmark)			100 µm strainer		MEM (Gibco, Taastrup, Denmark)	FBS	10%
				collagenase type I (Worthington Biochem, Freehold, NJ)	3 mg/mL					pencillin	25.000 IU/mL
				dispase II (Roche Diagnostics, Mannheim, Germany)	2.4 U/mL					streptomycin	25 mg/mL
Koyama 2009	14-35 y			collagenase type I	3 mg/mL	60 min	70 µm strainer		DMEM (Invitrogen Life Technologies, New York)	FBS (Equitech-Bio, Inc, Kerrbille, TX)	10%
										penicillin (Gibco)	100 U/mL
				dispase	4 mg/mL					streptomycin (Gibco)	100 mg/mL
Kraft 2010	21 y	Male		MEM (Gibco, Taastrup, Denmark)		30 min	100 µm strainer (BD Biosciences - Discovery Labware, Bedford, MA, USA)		MEM (Gibco, Taastrup, Denmark)	FBS (PAA Laboratories, Linz, Austria)	10%
	20 y	Female		collagenase type I (Worthington Biochem, Freehold, NJ, USA)	3 mg/mL					streptomycin (DuraScan Medical Products, Odense, Denmark)	25 mg/mL
				dispase II (Roche Diagnostics, Mannheim, Germany)	2.4 U/mL					penicillin (DuraScan Medical Products, Odense, Denmark)	25.000 IU/mL
Laino 2006	19-37 y			penicillin	100 U/mL	60 min	70 µm Falcon strainers (Becton & Dickinson, Sunnyvale, CA)		α-MEM	FBS (Invitrogen Celbio Italy, San Giuliano Milanese, Milan, Italy)	22.5%
				strptomycin	100 µg/mL					2P-ascorbic acid (Invitrogen Celbio Italy, San Giuliano Milanese, Milan, Italy)	100 µM
				clarithromycin	500 µg/mL					penicillin (Invitrogen Celbio Italy, San Giuliano Milanese, Milanese, Milan, Italy)	100 U/mL
				PBS	4 mL					L-glutamine (Invitrogen Celbio Italy, San Giuliano, Milanese Milanese, Milan, Italy)	2 mM
				collagenase type I	3 mg/mL					streptomycin (Invitrogen Celbio Italy, San Giuliano Milanese, Milanese, Milan, Italy)	100 µg/mL
				dispase	4 mg/mL						
Laino 2005	30-45 y			penicillin (Invitrogen, Milan, Italy)	100 U/mL	60 min	70 µm Falcon strainers (Becton & Dickinson, Sunnyvale, CA)		α-MEM	FBS (Invitrogen, Milan, Italy)	20%
				streptomycin (Invitrogen, Milan, Italy)	100 µg/mL					2P-ascorbic acid (Invitrogen, Milan, Italy)	100 µM
				claritromycin (Invitrogen, Milan, Italy)	500 µg/mL					penicillin (Invitrogen, Milan, Italy)	100 U/mL
				PBS	4 mL 0.1 mol						
				collagenase type I	3 mg/mL					streptomycin (Invitrogen, Milan, Italy)	100 µg/mL
				dispase	4 mg/mL						

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Lee 2014	18-39 y			collagenase	3 mg/mL	60 min	70 mm strainer (BD)		DMEM	FBS (Atlanta Biological)	10%
				dispase	4 mg/mL					antibiotics	1%
Lee 2012	28 y	Male		collagenase type I (Sigma, St. Louis, Mo., USA)	4 mg/mL	60 min	70 µm strainer (BD Bioscience, San Jose, Calif., USA)	Minced	α- MEM (Gibco-Invitrogen, Carlsbad, Calif., USA)	FBS (Gibco-Invitrogen)	15%
	25 y	Female								L-ascorbic acid-2-phosphate (Sigma)	100 µM
				dispase (Sigma)	2 mg/mL					AA (Gibco-Invitrogen)	1%
Lee 2011 (a)				collagenase type I (Worthington, Lakewood, NJ)	3 mg/mL	60 min	70 µm strainer (BD Falcon, Franklin Lakes, NJ)		α-MEM (Hyclone, South Logan, UT)	FBS (Hyclone)	10%
										AA (Gibco, Grand Island, NY)	1%
Lee 2011 (b)	18-22 y		60 mm						DMEM	FBS	10%
Lee 2011 (c)				collagenase type I (Gibco)	1 mg/mL	60 min		Minced	α-MEM (Hyclone, Road Logan, UT)	FBS (Hyclone)	15%
										L-glutamine (Sigma–Aldrich, St. Louis, MO)	2 mM
				dispase (Gibco)	2.4 mg/mL					ascorbic acid 2-phosphate (Sigma–Aldrich, St. Louis, MO)	100 µM
										AA	1%
Lee 2011 (d)	18-35 y			collagenase type I (Worthington Biochem, Freehold, NJ, USA)	3 mg/mL	60 min	40 µm strainer (Falcon, BD Labware, Frankin Lakes, NJ, USA)		α- MEM (Gibco-BRL, Grand Island, NY, USA)	FCS (Equitech-Bio Inc, Kerville, TX, USA)	10%
										ascorbic acid 2-phosphate (WAKO, Tokyo, Japan)	100 µmol/L
				dispase (Boehringer, Mannheim, Germany)	4 mg/mL					glutamine	2mmol/L
										streptomycin (Biofluids, Rockville, MD, USA)	100 µg/mL
										penicillin (Biofluids, Rockville, MD, USA)	100U/mL
Lee 2010 (a)				collagenase (Sigma)	4 mg/mL	15 min			α-MEM (Invitrogen)	FBS (Invitrogen)	10%
				DNAse I in DMEM/F12	0.5 mg/mL					AA (Invitrogen)	1% (1000 U/mL)
Lee 2010 (b)	17-38 y	Male		penicillin (Gibco,USA)	100 U/mL	60 min	70 µm cell strainer (Becton & Dickinson, Sunnyvale, CA)		DMEM (Gibco, USA)	FBS (Gibco, Australia)	10%
		Female		PBS (Gibco,USA)	4 mL				α-MEM (Gibco,USA)	AA (Gibco, USA)	1%
									α-MEM (group SF, Ultraculture, Lonza, USA)		
Lee 2010 (c)	18-30 y			collagenase type I (Sigma, St. Louis, MO)	4 mg/mL			Minced			
				dispase (Sigma)	2 mg/mL						
Lee 2008				collagenase type I (Worthington Biochem, Freehold, NJ)	3 mg/mL	60 min			α-MEM	FBS	20%
										L - ascorbic acid 2-phosphate (Sigma-Aldrich)	100 µM
										L-glutamine (Sigma)	2 mM
				dispase (Gibco)	4 mg/mL					penicillin (Gibco)	100 units/mL
										streptomycin (Gibco)	100 µg/mL
Li 2011	19-22 y	Male		collagenase type I	3 mg/mL	40-60 min		Minced	α- MEM (Gibco BRL, Life Technologies B.V., Breda, Netherlands)	FBS (Gibco, ERL)	10%
				dispase	4 mg/mL					penicillin (Gibco, ERL)	100 U/mL
										streptomycin (Gibco, ERL)	100 U/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Lin 2011	25 y	Male		collagenase type I	3 mg/mL	60 min	70 µm strainer (Pharmingen; BD, San Jose, CA)		Low glucose DMEM (Gibco-Invitrogen, Life Technologies, Carlsbad, CA)	FBS (HyClone, Logan, UT)	10%
										penicillin (Biological Technologies, Beit Haemek, Israel)	100 U/mL
	18 y	Female		dispase	4 mg/mL					streptomycin (Biological Technologies, Beit Haemek, Israel)	100 U/mL
										L-glutamine (Gibco, Grand Island, NY)	2 mmol/L
Lindroos 2008	21-31 y			collagenase type I (Invitrogen, Paisley, Scotland, UK)	3 mg/mL				DMEM/F-12 (1:1) (Invitrogen)	FBS (Invitrogen)	10%
										L-ascorbic acid phosphate (Sigma–Aldrich, MO, USA)	100 µM
										L-glutamine (GlutaMAX I; Invitrogen)	100 µM
				dispase (Invitrogen)	4 mg/mL					penicillin (Invitrogen)	100 U/mL
										streptomicin (Invitrogen)	0.1 mg/mL
										amphotericin B (Invitrogen)	0.25 µg/mL
Liu 2014				collagenase type I	3 mg/mL				α-MEM (Gibco BRL, Grand Island, NY, USA)	FCS (Equitech-Bio Inc.,Kerrville, TX, USA),	20%
										L-ascorbic acid 2-phosphate (Wako, Tokyo, Japan)	100 µM
										L-glutamine (Biofluids Inc., Rockville, MD, USA)	2 mM
				dispase	4 mg/mL					penicillin (Biofluids Inc., Rockville, MD, USA)	100 U/mL
										streptomycin (Biofluids Inc., Rockville, MD, USA)	100 µg/mL
Luo 2014 (a)	18-25 y			collagenase type I (Sigma-Aldrich)	3 mg/mL	30 min		Minced	α- MEM	FBS (Hyclone, Logan, UT)	15%
										penicilin G (Invitrogen)	100 U/mL
				dispase (Sigma-Aldrich)	4 mg/mL					streptomycin (Invitrogen)	100 mg/mL
Luo 2014 (b)				collagenase type I (Sigma)	3 mg/mL	45-60 min	70 µm cell strainer (Becton/Dickinson, Franklin Lakes, NJ, USA)	Minced	α- MEM	FBS (Hyclone)	15%
										L-glutamine (Invitrogen)	2 mmol/L
										penicilin G (Sigma)	100 U/mL
				dispase (Sigma)	4 mg/mL					streptomycin (Sigma)	100 mg/mL
										ascorbic acid (Sigma)	50 mg/mL
Ma 2012	18-28 y			collagenase type I (Invitrogen Life Technology, Carlsbad, CA)	3 mg/mL	60 min	70 µm cell strainer (Carrigtwohill Co, Cork, Ireland)	Minced	DMEM (Gibco, Life Technologies, Grand Island, NY)	FBS	15%
										penicillin	100 U/mL
				dispase (Sigma, St Louis, MO)	4 mg/mL					streptomycin	100 mg/mL
										ascorbic acid	50 mg/mL
Makino 2013				collagenase type I (Worthington Biochemicals, Lakewood, NJ,	0.3%	60 min	70 µm cell strainer (BD Biosciences, San Jose, CA, USA)		α-MEM (Invitrogen, Carlsbad, CA, USA)	FBS (Equitech-Bio, Kerrville, TX, USA)	15%
										L-glutamine (Nacalai Tesque, Kyoto, Japan)	2 mM
				dispase II (Sanko Junyaku, Tokyo, Japan)	0.4%					penicillin (Nacalai Tesque)	100 U/mL
										streptomycin (Nalacai Tesque)	100 µg/mL

Author and year	Age	Gender	Isolation techniques					Culture Medium	Medium supplementation	
			Explant	Enzymatic			Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering			
Mangano 2011				collagenase type I	3 mg/mL	60 min	70 µm Falcon strainers (BD, Franklin Lakes, NJ, USA)		MegaCell (Sigma, Milan, Italy)	FBS	10%
				dispase	4 mg/mL					2P-ascorbic acid (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mM
				penicillin	100 U/mL					L-glutamine (Invitrogen, San Giuliano Milanese, Milan, Italy)	2 mM
				streptomycin	100 mg/mL					penicillin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 U/mL
										streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mg/mL
Mangano 2010				collagenase type I (Invitrogen, San Giuliano Milanese, Milan,	3 mg/mL	60 min	70 µm Falcon strainers (BD, Franklin Lakes, NJ, USA)		MegaCell (Sigma, Milan, Italy)	FBS	10%
				dispase (Invitrogen, San Giuliano Milanese, Milan, Italy)	4 mg/mL					2P-ascorbic acid (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mM
				PBS (Invitrogen, San Giuliano, Milanese, Milan, Italy)						L-glutamine (Invitrogen, San Giuliano Milanese, Milan, Italy)	2 mM
				penicilin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 U/mL					penicillin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 U/mL
				streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mg/mL					streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mg/mL
Manikandhan 2010				collagenase type I		15-30 min	70 µm cell strainer		DMEM/Ham'sF12	FBS	15%
										penicillin	100 U/mL
										streptomycin	100 µg/mL
				collagenase type II						L - glutamine	2 mM
										non essencial amino acids	2 mM
Marchionni 2009	Mean of 35 y			collagenase type I	1 mg/mL	30 min		Minced	DMEM	FBS	10%
										penicillin	100 U/mL
										streptomycin	100 µg/mL
										amphotericin B	0.25 µg/mL
Martens 2012	18-24 y		1 mm³						α-MEM (Gibco-Invitrogen Corp.,Paisley, Scotland, UK)	FCS (Biochrom AG, Berlin, Germany)	10%
										L - glutamine (Gibco-Invitrogen Corp.)	2 mM
										penicillin (Gibco-Invitrogen Corp.)	100 U/mL
										streptomycin (Gibco-Invitrogen Corp.)	100 µg/mL
Martin 2013	Mean of 22.5 y			collagenase type I (Gibco BRL Life Technologies, Karlsruhe, Germany)	2 mg/mL	360 min			DMEM (Sigma-Aldrich)	FBS (Sigma-Aldrich)	10%
Min 2011	20-25 y		Realized (not described)						α-MEM (Gibco)	FBS (Hyclone)	20%
										antibiotics	

Author and year	Age	Gender	Isolation techniques					Culture Medium	Medium supplementation	
			Explant	Enzymatic			Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering			
Mokry 2010	18-27 y	Male		collagenase type I (Sevapharma, Czech Republic)	3 mg/mL			α- MEM (Gibco, UK)	FCS (PAA, USA)	2%
									EGF (PeproTech, USA)	10 ng/mL
									PDGF (PeproTech, USA)	10 ng/mL
									L-ascorbic acid (Sigma, USA)	
									glutamine (Gibco, UK)	2%
		Female		dispase (Gibco, UK)	4 mg/mL				penicillin (Gibco, UK)	
									streptomycin (Gibco, UK)	
									gentamycin (Gibco, UK)	
									dexamethasone (Sigma, USA)	
									ITS (Sigma)	10 µl/mL
Mori 2011				collagenase type I (Gibco Ltd., Uxbridge, UK)	3 mg/mL	60 min	70 µm BD Falcon strainer (Becton & Dickinson, Sunnyvale, CA)		FBS (Gibco Ltd.)	10%
				dispase (Gibco Ltd., Uxbridge, UK)	4 mg/mL			α-MEM (Gibco Ltd.)	penicillin-G (Gibco Ltd.)	100 U/mL
									streptomycin (Gibco Ltd.)	100 µg/mL
									fungizone (Gibco Ltd.)	0.25 µg/mL
Murakami 2013	18-29 y			Realized (not described)				Minced	DMEM (Sigma Aldrich, St. Louis, MO)	human serum 10%
Murakami 2012	18-29 y			Realized (not described)			40 µm BD Falcon strainer	Minced	EBM2 (Cambrex Bio Science, Walkersville, MD)	FBS (Invitrogen Corp) 10%
										IGF(Cambrex Bio Science) 10 ng/mL
										EGF (Cambrex Bio Science) 5 ng/mL
Muthna 2010	12-23 y	Male		collagenase (Sevapharma, Praha, Czech Republic)		70 min			α- MEM (Invitrogen)	FCS (PAA, Dartmouth, MA) 2%
										EGF (PeproTech, Rocky Hill, NJ) 10 ng/mL
										PDGF (PeproTech) 10 ng/mL
										L -ascorbic acid (Sigma, St. Louis. MO)
										glutamine (Invitrogen) 2%
		Female		dispase (Invitrogen, Carlsbad, CA)						streptomycin (Invitrogen)
										penicillin (Invitrogen)
										gentamycin (Invitrogen)
Nadeem 2013			1x1x2 mm³						α-MEM (Lonza)	FBS (Lonza) 10%
										penicillin (Gibco) 100 U/mL
										streptomycin (Gibco)
Nakamura 2009				collagenase type I	3 mg/mL	60 min	70 mm cell strainers (Falcon; BD Labware, Franklin Lakes, NJ)		DMEM (Gibco, Rockville, MD)	mesenchymal cell growth supplement (Lonza Inc, 20%
										Walkersville, MD)
										penicillin (Gibco) 100 U/mL
				dispase	4 mg/mL					streptomycin (Gibco) 100 µg/mL
										amphotericin B (Gibco) 0.25 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration		Filtering				
Nam 2011	19-25 y			collagenase type I	3 mg/mL	60 min	70 µm strainer	Minced	α-MEM (Gibco, USA)	FBS (Gibco, USA)	10%
										penicillin (Gibco, USA)	100 U/mL
				dispase	4 mg/mL					streptomycin (Gibco, USA)	100 µg/mL
Navabazam 2013	15-32 y			collagenase type I	3 mg/mL	60 min	70 mm cell strainer (Falcon, BD Labware, Franklin Lakes, NJ)		α-MEM (Gibco BRL, Carlsbad, CA)	FBS (Gibco BRL)	10%
										penicillin (Gibco BRL)	100 U/mL
				dispase	4 mg/mL					streptomycin (Gibco BRL)	100 U/mL
										amphotericin B (Biochrom AG)	100 mg/mL
Nawi 2013				collagenase type I		60 min		Minced	DMEM (Gibco BRL)	FBS (Equitech-Bio, Kerrville, TX)	10%
										L-ascorbic acid 2-phosphate (Wako Pure Chemicals, Osaka)	100 µm
										L-glutamine (Biofluids, Rockville, MD)	2 mM
										penicillin (Biofluids, Rockville, MD)	100 U/mL
										streptomycin (Biofluids, Rockville, MD)	100 mg/mL
Nesti 2011	18-35 y		Realized (not described)						α-MEM (Sigma Aldrich, Germany)	FBS (Sigma)	20%
										penicillin (Pharmacia &UpJohn SpA, Italy)	100 IU/mL
										streptomycin (Bristol-Myers Squibb SpA, Italy)	100 IU/mL
										L-glutamine (Cambrex Bioscience Inch., Baltimore)	2 mM
Neuss 2008				collagenase type I (Sigma, Steinheim, Germany)	3 mg/mL	60 min	70 µm strainer (BD, Heidelberg, Germany)		α-MEM (Gibco, Karlsruhe, Germany)	FCS (PAA, Colbe, Germany)	20%
										L-ascorbic-acid-2-phosphate (Sigma, Steinheim, Germany)	100 µM
				dispase (Sigma, Steinheim, Germany)	4 mg/mL					gentamycine (Gibco, Karlsruhe, Germany)	50 µg/mL
Niu 2014	18-25 y			collagenase type I (Gibco, Gaithersburg, MD, USA)	3 mg/mL	120 min	70 µm strainer (BD Falcon, Franklin Lakes, NJ, USA)	Minced	α- MEM (Gibco)	FBS (Gibco)	20%
										penicillin	100 U/mL
				dispase (Gibco, Gaithersburg, MD, USA)	4 mg/mL					streptomycin	100 µg/mL
Oancea 2013	12-17 y			collagenase IA solution	3 mg/mL	120 min		Minced - 0.5 mm^3	Mesencult (Stem Cell Technology)		
Okamoto 2009	22-26 y			collagensase type I (Invitrogen, Carlsbad, CA)	3 mg/mL	60 min	70 µm cell strainer	Minced	α- MEM (Sigma Chemical Company, St Louis, MO)	FBS (Cancera International Inc, Ontario, Canada)	15%
										L-ascorbic acid 2-phosphate (Sigma Chemical Company)	100 mmol/L
										L-glutamine (Sigma Chemical Company)	2 mmol/L
				dispase (Invitrogen)	4 mg/mL					penicillin (Meiji Seika, Tokyo, Japan)	100 U/mL
										streptomycin (Meiji Seika)	100 mg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Osathanon 2014				collagenase type I (Gibco, Carlsbad, CA, USA)				Minced	DMEM (Gibco)	FBS (Gibco)	10%
										L- glutamine (Gibco)	2 mM/L
										penicillin (Gibco)	100 U/mL
										streptomycin (Gibco)	100 µg/mL
										amphotericin B (Gibco)	5 µg/mL
Osathanon 2011				collagenase type I (Gibco, USA)				Minced	DMEM (Gibco)	FBS (Gibco)	10%
										L-glutamine (Gibco)	2 mM
										penicillin (Gibco)	100 U/mL
										streptomycin (Gibco)	100 µg/mL
										amphotericin B (Gibco)	5 µg/mL
Paino 2010				collagenase type I	3 mg/mL	60 min	70 µm Falcon strainers (Becton & Dickinson, Sunnyvale, CA, USA)		MegaCell (Sigma, St. Louis, MO, USA)	FBS (Invitrogen, Carlsbad, CA, USA)	10%
										L-ascorbic acid 2-phosphate (Invitrogen, Carlsbad, CA, USA)	100 µM
										L-glutamine (Invitrogen, Carlsbad, CA, USA)	2 mM
				dispase	4 mg/mL					penicillin (Invitrogen, Carlsbad, CA, USA)	100 U/mL
										streptomycin (Invitrogen, Carlsbad, CA, USA)	100 µg/mL
Palumbo 2013				collagenase type I	3 mg/mL	60 min	100 µm Falcon Cell Strainers (Sigma, USA)		α-MEM (Sigma, USA)	FBS (Sigma, USA)	20%
										penicillin (Sigma, USA)	100 U/mL
				dispase	4 mg/mL					streptomycin (Sigma, USA)	100 µg/mL
Pang 2013	16-22 y		1 mm^3						α-MEM (Invitrogen, Carlsbad, CA)	FBS (Invitrogen)	10%
										glutamine (Wako, Tokyo, Japan)	2 mmol/L
										ascorbic acid-2-phosphate (Wako, Tokyo, Japan)	100 µmol/L
										penicillin (Biofluids, Rockville, MD)	100 U/mL
										streptomycin (Biofluids, Rockville, MD)	100 µg/mL
Papaccio 2006	21-45 y			collagenase type I	3 mg/mL	60 min	70 µm Falcon strainers (Becton & Dickinson, Franklin Lakes, NJ)		α-MEM	FCS (Invitrogen, San Giuliano Milanese, Milan, Italy)	20%
				dispase	4 mg/mL					2P-ascorbic acid (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mM
				penicillin	100 U/mL					L-glutamine (Invitrogen, San Giuliano Milanese, Milan, Italy)	2 mM
				streptomycin	100 µg/mL					penicillin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 U/mL
										streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 µg/mL
Park 2013	18-35 y			collagenase type I (Worthington Biochemical Corp., NJ, USA)	3 mg/mL	60 min	40 µm strainer (BD Falcon ™, BD Labware, CA, USA)		α-MEM (Gibco BRL, Life Technologies, NY, USA)	FCS (Gibco BRL)	10%
										ascorbic acid 2-phosphate (Wako Chemicals USA, VA, USA)	100 µmol/L
										glutamine (Sigma-Aldrich, MO, USA)	2 mmol/L
				dispase (Boehringer Mannheim, QC, Canada)	4 mg/mL					penicillin (Sigma-Aldrich)	100 U/mL
										streptomycin (Sigma-Aldrich)	100 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Patil 2014	16-18 y	Male		collagenase type I (Sigma, Chemical Company, St.Louis, MO,USA)	1 mg/mL	30 min	100 µm nylon cell strainer	Minced	ADMEM (Gibco, Life Technologies,Grand Island,NY,USA)	FBS (Sigma Chemical Company, St.Louis,MO,USA)	10%
										glutamine (Sigma Chemical Company, St.Louis,MO,USA)	4 mM
										penicillin (Sigma Chemical Company, St.Louis,MO,USA)	10.000 IU (1%)
										streptomycin (Sigma Chemical Company, St.Louis,MO,USA)	10.000 µg/mL (1%)
Pereira 2012 (a)	17-43 y			collagenase type I (Gibco)	3 mg/mL	60 min			α-MEM (Gibco, Gaithersburg, MD)	FBS (Gibco)	10%
										penicillin (Gibco)	100 U/mL
										streptomycin (Gibco)	100 mg/mL
Pereira 2012 (b)	17-43 y			collagenase type I (Gibco)	3 mg/mL	60 min			α-MEM (Gibco, Gaithersburg, MD)	FBS (Gibco, Grand Island, NY, USA)	10%
										penicillin (Gibco, Rockville, MD, USA)	100 U/mL
										streptomycin (Gibco, Rockville, MD, USA)	100 mg/mL
Perry 2008	18-30 y			collagenase type I with thermolysin as the neutral protease (Vitacyte, Indianapolis, IN)		40 min			Mesencult complete medium (StemCell Technologies)	amphotericin B (JR Scientific, Woodland, CA)	0.25 µg/mL
				collagenase type II with thermolysin as the neutral						penicillin-G (JR Scientific, Woodland, CA)	100 IU/mL
										streptomycin (JR Scientific, Woodland, CA)	100 µg/mL
Picchi 2013			Realized (not described)						α-MEM (Sigma Aldrich)	FBS (Sigma Aldrich)	20%
										penicillin (Pharmacia & Upjohn SpA, Milano, Italy)	100 IU/mL
										L-glutamine (Cambrex Bioscience, Inc., Baltimore, MD)	2 mM
Pierdomenic 2005	Mean of 40 y	Male	Realized (not described)						MEM (Sigma)	inactivated FBS (Sigma)	25%
										penicillin (Sigma)	200 U/mL
		Female								streptomycin (Sigma)	200 µg/mL
										amphotericin B (Sigma)	2.5 µg/mL
Ponnaiyan 2012	18-22 y			collagenase type I	2 mg/mL	60 min		Minced - 0.5 mm^3	DMEM	FBS	15%
										penicillin G	100 U/mL
				dispase	4 mg/mL					streptomycin	100 µg/mL
Riccio 2010	18-35 y			collagenase type I (Sigma, St. Louis, MO, USA)	3 mg/ml	60 min	100 µm falcon cell strainers		α-MEM (Sigma, St. Louis, MO, USA)	FBS (Euroclone, Milano, Italy)	20%
										2P-ascorbic acid (Sigma, St. Louis, MO, USA)	100 µM
										L-glutamine (Sigma, St. Louis, MO, USA)	2 mM
				dispase (Sigma, St. Louis, MO, USA)	4 mg/ml					penicillin (Sigma, St. Louis, MO, USA)	100 U/mL
										streptomycin (Sigma, St. Louis, MO, USA)	100 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Rizk 2013 (a)				collagenase		15 min		Minced - 2 mm	α- MEM	FBS	10%
				dispase						Antibiotics	
Risk 2013 (b)				collagenase		15 min		Minced 2 mm	α- MEM	FBS	10%
				dispase						Antibiotics	
Rodriguez-Lozano 2013				collagenase type I (Sigma Aldrich, St. Louis, MO, USA)	3 mg/mL	60 min			DMEM	FCS	10%
										penicillin	100 U/mL
										streptomycin	100 µg/mL
Rodríguez-Lozano 2012	Mean of 29 y, 21-45 y	Male		collagenase type I (Sigma Aldrich, St. Louis, MO)	3 mg/mL	60 min					
		Female									
Ryu 2009				collagenase type I (Sigma)	4 mg/mL				α-MEM (Invitrogen)	FBS (Invitrogen)	10%
				DNAse I	0.5 mg/mL					AA (Invitrogen)	1000 U/mL (1%)
Sakai 2012	18-30 y			collagenase type I	3 mg/mL	60 min			DMEM	FCS	10%
				dispase	4 mg/mL						
Schiraldi 2012	21-45 y			collagenase type I	3 mg/mL	60 min	70 µm Falcon strainers (Becton & Dickinson,Milan, Italy)		DMEM	FBS (Lonza, Milan, Italy)	10%
										2P-ascorbic acid (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 mM
										L-glutamine (Invitrogen, San Giuliano Milanese, Milan, Italy)	2 mM
				dispase	4 mg/mL					penicillin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 U/mL
										streptomycin (Invitrogen, San Giuliano Milanese, Milan, Italy)	100 µg/mL
Seifrtova 2013				collagenase (Sevapharma, Prague, Czech Republic)		70 min			α-MEM (Invitrogen)	FCS (PAA, Dartmouth, NH, USA)	2%
										EGF(PeproTech, Rocky Hill, NJ, USA)	10 ng/mL
										PDGF (PeproTech, Rocky Hill, NJ, USA)	10 ng/mL
										L-ascorbic acid (Sigma, St Louis, MO, USA)	
										glutamine (Invitrogen)	2%
				dispase (Invitrogen, Carlsbad, CA, USA)						streptomycin (Invitrogen)	
										penicillin (Invitrogen)	
										gentamycin (Invitrogen)	
										dexamethasone (Sigma)	50 nmol/L

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Seifrtova 2012				collagenase (Sevapharma, Prague, Czech Republic)		70 min			α-MEM (Invitrogen)	FCS (PAA, Dartmouth, NH, USA)	2%
										EGF(PeproTech, Rocky Hill, NJ, USA)	10 ng/mL
										PDGF (PeproTech, Rocky Hill, NJ, USA)	10 ng/mL
										L-ascorbic acid (Sigma, St Louis, MO, USA)	
										glutamine (Invitrogen)	2%
				dispase (Invitrogen, Carlsbad, CA, USA)						streptomycin (Invitrogen)	
										penicillin (Invitrogen)	
										gentamycin (Invitrogen)	
										dexamethasone (Sigma)	50 nmol/L
Seo 2013	20-28 y			collagenase type I (Sigma-Aldrich, St Louis, MO)	3 mg/mL	10 min		Minced	high-glucose DMEM (Invitrogen,Carlsbad, CA)	FBS (Invitrogen)	10%
										streptomycin	100 mg/mL
				dispase (Stem cell Technologies, Vancouver, BC, Canada)	5 mg/mL					penicillin	100 U/mL
Shafiei 2014	20-25 y			collagenase type I (Sigma Aldrich, St Louis, MO)	3 mg/mL	30-60 min			DMEM (Gibco-Invitrogen, Carlsbad, CA,USA)	FBS (Gibco/Invitrogen, Carlsbad, CA,USA)	20%
										glutaMAX (Gibco-Invitrogen, Carlsbad, CA,USA)	4 mM
				dispase (Sigma-Aldrich, St. Louis, MO)	4 mg/mL					penicillin (Gibco-Invitrogen, Carlsbad, CA,USA)	100 U/mL
										streptomycin (Gibco-/Invitrogen, Carlsbad, CA,USA)	100 µg/mL
Shekar 2012				collagenase (filtered; CLS1™; Worthington Biochemical Corporation)	2 mg	60 min		Minced	α-MEM serum free	penicillin	100 IU
				dispase (neutral protease, grade II; Roche)	1 mg					streptomycin	100 µg/mL
Shi 2002				collagenase type I							
				dispase							
Sollazzo 2011	20-25 y			collagenase type I (Sigma)	3 mg/mL	60 min	70 µm Falcon strainers (Sigma Aldrich, Inc., St Louis, Mo, USA)		α-MEM (Sigma Aldrich, Inc., St Louis, Mo, USA)	FCS (Sigma)	20%
				dispase (Sigma)	4 mg/mL					2P-ascorbic acid (Sigma)	100 µM
				penicillin (Sigma)	100 U/mL					L-glutamine (Sigma)	2 mM
				streptomycin (Sigma)	100 µg/mL					penicillin (Sigma)	100 U/mL
				claritromycin (Sigma)	500 µg/mL					streptomycin (Sigma)	100 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Son 2006				collagenase type I (Worthington Biochem, Freehold, NJ)	3 mg/mL	60 min			α-MEM (Gibco)	FBS (Sigma-Aldrich)	20%
										L-ascorbic acid 2-phosphate (Sigma-Aldrich)	100 µM
										L-glutamine (Sigma)	2 mM
				dispase (Gibco)	4 mg/mL					penicillin (Gibco)	100 U/mL
										streptomycin (Gibco)	100 µg/mL
Spath 2010	22-35 y		1-2 mm³	trypsin (Invitrogen)	0.2%	5 min			DMEM	FCS	10%
										penicillin	100 U/mL
										streptomycin	100 µg/mL
										L-glutamine	2 mM
										β-mercaptoethanol	0.1 mM
Stevens 2008				collagenase type I	3 mg/mL	60 min	70 µm pore size strainer		DMEM	FCS	10%
										streptomycin (Invitrogen, Cergy Pontoise, France)	1%
				dispase	4 mg/mL					penicillin (Invitrogen, Cergy Pontoise, France)	1%
										vitamin C L-ascorbic acid 2-phosphate (Sigma Aldrich Inc, USA)	100 µM
Stokowski 2007	18-40 y			collagenase type I (Worthington Biochem, Freehold, NJ)	3 mg/mL	60 min	70 µm strainer (Falcon; BD Labware, Franklin Lakes, NJ)		α-MEM (SAFC, Lenexa, KS)	FCS (SAFC)	20%
										L-ascorbic acid 2-phosphate (Wako Chemical, Tokyo)	100 µM
										L-glutamine (SAFC, Lenexa, KS)	2 mM
				dispase (Boehringer Mannheim, Mannheim, Germany)	4 mg/mL					penicillin (SAFC, Lenexa, KS)	100 U/mL
										streptomycin (SAFC, Lenexa, KS)	100 µg/mL
Struys 2013	16-19 y		Realized (not described)						α-MEM (Gibco, Invitrogen Corp., Paisley, UK)	FBS (Biochrom AG., Berlin, Germany)	10%
										penicillin (Gibco, Invitrogen Corp., Paisley, UK)	1%
										streptomycin (Gibco, Invitrogen Corp., Paisley, UK)	1%
										fungizone (Gibco, Invitrogen Corp., Paisley, UK)	0.2%
										L-glutamine (Gibco, Invitrogen Corp., Paisley, UK)	2 mM
										ascorbic acid (Gibco, Invitrogen Corp., Paisley, UK)	50 µg/mL
Struys 2011			1-2 mm³						BME (Gibco, Invitrogen Corp., Paisley, UK)	FBS (Biochrom AG., Berlin, Germany)	10%
										penicillin (Gibco, Invitrogen Corp., Paisley, UK)	1%
										streptomycin (Gibco, Invitrogen Corp., Paisley, UK)	1%
										fungizone (Gibco, Invitrogen Corp., Paisley, UK)	0.2%
										L-glutamine (Gibco, Invitrogen Corp., Paisley, UK)	2 mM
										ascorbic acid (Gibco, Invitrogen Corp., Paisley, UK)	50 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Suchanek 2013	23 y	Male		collagenase (Sevapharma, Czech Republic)		70 min			α-MEM (Gibco, UK)	FCS (PAA, USA)	2%
										EGF (PeproTech, USA)	10 ng/mL
										PDGF (PeproTech, USA)	10 ng/mL
										L–ascorbic acid (Sigma,USA)	0.2 mM
										glutamine (Gibco, UK)	2%
	22 y	Female		dispase (Gibco, UK)						penicillin (Gibco, USA)	100 U/mL
										streptomycin (Gibco, USA)	100 µg/mL
										dexamethasone (Sigma, USA)	50 nM
										ITS (Sigma, USA)	10 µl/mL
Suchanek 2009	12-23 y	Male		collagenase (Sevapharma, CR)		70 min			α-MEM (Gibco, UK)	FCS (PAA, USA)	2%
										EGF (PeproTech, USA)	10 ng/mL
										PDGF (PeproTech, USA)	10 ng/mL
										L–ascorbic acid (Sigma,USA)	
										glutamine (Gibco, UK)	2%
		Female		dispase (Gibco, Scotland)						penicillin (Gibco, USA)	100 U/mL
										streptomycin (Gibco, USA)	100 µg/mL
										dexamethasone (Sigma, USA)	50 nM
										ITS (Sigma, USA)	10 µl/mL
Suchanek 2007	15-23 y			collagenase (Sevapharma, CR)		70 min			α-MEM (Gibco, UK)	FCS (PAA, USA)	2%
										EGF (PeproTech, USA)	10 ng/mL
				dispase (Gibco, Scotland)						PDGF (PeproTech, USA)	10 ng/mL
										dexamethasone (Sigma, USA)	50 nM
Suh 2014	19-40 y			collagenase type I (Gibco™, Grand Island, NY, USA)	3 mg/mL	60 min	70 µm cell strainer (BD Falcon™, Franklin Lakes, NJ, USA)		α-MEM (Gibco™, Grand Island, NY, USA)	FCS (Gibco™, Grand Island, NY, USA)	10%
										ascorbic acid 2-phosphate (Sigma-Aldrich®, St. Louis, MO, USA)	10 mM
				dispase (Boehringer, Mannheim, Germany)	4 mg/mL					glutamine (Gibco™, Grand Island, NY, USA)	2 mM
										penicillin (Gibco™, Grand Island, NY, USA)	100 U/mL
										streptomycin (Gibco™, Grand Island, NY, USA)	100 mg/mL
Suri 2008			Realized (not described)						α-MEM (BioWhittaker, Walkersville,MD, USA)	FBS (Invitrogen, Grand Island, NY, USA)	10%
										L-glutamine (Sigma, St. Louis, MO, USA)	2 mM
										penicillin (Sigma, St. Louis, MO, USA)	100 IU/mL
										streptomycin (Sigma, St. Louis, MO, USA)	100 µg/mL

Author and year	Age	Gender	Isolation techniques					Culture Medium	Medium supplementation	
			Explant	Enzymatic			Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering			
Suzyki 2011	14 y	Male		collagenase type I (Invitrogen, Carlsbad, CA, USA)	3 mg/mL		70 µm strainer (BD, Franklin Lakes, NJ, USA)		DMEM (Sigma-Aldrich, St. Louis, MO, USA)	FBS (Biocell, Rancho Dominguez, CA, USA)	10%
	28 y	Female		dispase (Invitrogen, Carlsbad, CA, USA)	4 mg/mL					AA (Invitrogen)	1%
Tamaki 2013	16-28 y			collagenase type I (Sigma, St Louis, MO)	3 mg/mL	60 min	70 µm cell strainer (Falcon, BD Labware, Franklin Lakes, NJ)	Minced	DMEM/F12 (Gibco BRL, Carlsbad, CA)	FBS (Sigma)	15%
				dispase (Sanko Pure Chemical Ltd., Tokyo, Japan)	4 mg/mL					glutamine (GlutaMAX I - Invitrogen, Carlsbad, CA)	2 mM
										penicillin (Gibco BRL)	100 U/mL
										streptomycin (Gibco BRL)	100 µg/mL
Tammaro 2014	18-22 y			collagenase type I (Sigma Aldrich, Italy)	3 mg/mL	30 min	70 µm cell strainer (Falcon, Italy)	Minced	α-MEM (Hyclone, Italy)	FBS (Hyclone, Italy)	15%
										L-glutamine (Hyclone, Italy)	2 mM
										L-ascorbic acid-2-phospate (Hyclone, Italy)	100 mM
				dispase (Sigma–Aldrich, Italy)	4 mg/mL					penicillin G (Hyclone, Italy)	100 U/mL
										streptomycin (Hyclone, Italy)	100 µg/mL
										fungizone (Hyclone, Italy)	0.25 µg/mL
Tandon 2010				trypsin (Invitrogen)	0.05%	10-20 min		Minced	DMEM-F12 (Invirtogen)	FBS (Hyclone)	10%
										ascorbic acid	100 µM
										L-Glutamax	2 mM
										penicillin	100 U/mL
										streptomycin	100 µg/mL
Tirino 2012				collagenase type I (Gibco, Invitrogen)	3 mg/mL	60 min	70 µm Falcon strainers (Becton & Dickinson)		Mega Cell (Sigma)	FBS (Gibco, Invitrogen)	10%
										2P-ascorbic acid (Sigma)	100 µM
				dispase (Gibco, Invitrogen)	4 mg/mL					L -glutamine (Lonza)	2 mM
				gentamicin (Sigma)						penicillin (Lonza)	100 U/mL
										streptomycin (Lonza)	100 µg/mL
Tomic 2011				collagenase type I (Sigma)	1 mg/mL	60 min		Minced	DMEM (Sigma, Munich, Germany)	FCS (Sigma)	10%
										penicillin (Galnika)	1%
				DNAase	25 µg/mL					streptomycin (Galenika)	1%
										gentamicin (Panfarma)	1%
Tom-Kun 2011				collagenase type I (Sigma, St Louis, MO)	3 mg/mL	30-60 min	70 µm cell strainer (Becton/Dickinson, Franklin Lakes, NJ)	Minced - 2x2x1 mm	α-MEM (Invitrogen, Carlsbad, CA)	FBS (Invitrogen)	15%
										penicillin-G (Invitrogen)	100 U/mL
				dispase (Sigma, St Louis, MO)	4 mg/mL					streptomycin (Invitrogen)	100 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Trubiani 2012			Realized (not described)						MSCM (Cambrex Co., Walkersville, MD, USA)		
Trubiani 2010			Realized (not described)						MSCM (Cambrex Co., Walkersville, MD, USA)		
Trubiani 2007	24-30 y		Realized (not described)						MSCM (Cambrex Co., Walkersville, MD, USA)		
Uchiyama 2009	37 and 42 y	Female		collagenase (Wako, Osaka, Japan)	0.2 %	15 min			α- MEM (Invitrogen, Carlsbad, CA, USA)	FBS	15%
Um 2011	20-24 y	Male		collagenase type I (Bio Basic Inc., Toronto, Canada)	3 mg/mL	90 min	70 µm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA)		α- MEM (Gibco BRL)	FBS (Gibco BRL)	15%
										L-ascorbic acid (Gibco BRL)	100 µM
				dispase (Gibco BRL, Grand Island, NY, USA)	4 mg/mL					L-glutamine (Gibco BRL)	2 mM
										AA (Gibco BRL)	100 U/mL
Vandomme 2014				collagenase type I (Invitrogen)	3 mg/mL	60 min	70 µm pore strainer (BD Biosciences)	Minced	DMEM (Glutamax)	FCS (Lonza)	10%
										glucose (Invitrogen)	1 mg/mL
										streptomycin solution (Invitrogen)	1%
				dispase (Invitrogen)	4 mg/mL					penicillin solution (Invitrogen)	100 mM
										vitamin C (Sigma-Aldrich)	
Varga 2011	Mean of 27 y	Male		collagenase type I (PAA, Austria)	0.1%	60 min			α-MEM (PAA, Austria)	FBS (PAA, Austria)	10%
		Female								gentamicin	160 µg/mL
Vasandan 2014	17-28 y			collagenase Blend type H (Sigma-Aldrich, St. Louis, MO, USA)	0.5 mg/mL	Overnight			KO-DMEM (Gibco)	FBS (HyClone, Thermo Scientific, Mordialloc, Vic., Australia)	10%
										glutamax (Gibco)	1 X
										AA	1 X
Ventura 2007			Cut into fragments						DMEM (BioWhittaker Cambrex, Walkersville, MD)	FBS (BioWhittaker Cambrex, Walkersville, MD)	25%
										penicillin (Sigma)	200 U/mL
										streptomycin (Sigma)	100 µg/mL
										amphotericin B (Sigma)	2.5 µg/mL
Vishwanath 2013	Less than 25 y			collagenase	3 mg/mL	60 min			DMEM (Gibco, Invitrogen)	FBS (HyClone, USA)	10%
				dispase	4 mg/mL					AA (Gibco, Invitrogen Corporation)	5 X
										L-glutamine	5 mM

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Wada 2009				collagenase type I (Worthington Biochemical, Lakewood, NJ)	3 mg/mL	120 min			α-MEM (Sigma Aldrich, St. Louis, MO)	FCS (Thermo Electron., Melbourne, Australia)	10%
										L-glutamine (Sigma–Aldrich)	2 mM
										L-ascorbate-2-phosphate (Wako Pure Chemical Industries, Richmond, VA)	100 µM
				dispase type II (Roche Diagnostics, Indianapolis, IN)	4 mg/mL					streptomycin (JRH Biosciences)	50 µg/mL
										sodium pyruvate (Sigma–Aldrich)	1 mM
										penicillin G (JRH Biosciences, Lenexa, KS)	50 U/mL
Wang 2014	19-28 y			collagenase type I (Sigma, USA)	3 mg/mL	30 min	70 µm strainer	Minced	α-MEM (Hyclone, USA)	FBS (Hyclone, USA)	10%
										penicillin (Sigma, USA)	100 U/mL
				dispase (Sigma, USA)	4 mg/mL					streptomycin (Sigma, USA)	100 µg/mL
Wang 2013 (a)	14-25 y			collagenase type I	3 mg/mL	60 min	70 µm strainer		DMEM/F12 (Hyclone, Logan, UT, USA)	FBS (Hyclone)	20%
				dispase	4 mg/mL					penicillin (Beyotime Institute of Biotechnology, Beijing, China)	100 U/mL
				PBS	1 X					streptomycin (Beyotime Institute of Biotechnology, Beijing, China)	100 µg/mL
Wang 2013 (b)	12-13 y			collagenase type I (Sigma,St. Louis, MO, USA)	3 mg/mL	60 min		Minced - 1 mm^3	phenol red free L-DMEM (Gibco, Life Technologies, Grand Island, NY, USA)	FBS (Hyclone, Logan, UT, USA)	10%
										penicillin	100 U/mL
				dispase (Sigma)	4 mg/mL					streptomycin	100 µg/mL
Wang 2012	18-20 y			collagenase type I (Sigma, St. Louis, MO, USA)	0.3 mg/mL	60 min			DMEM (Gibco, Life Technologies, Grand Island, NY, USA)	FBS (Gibco-BRL, NY, USA)	15%
										penicillin (Sigma–Aldrich, St. Louis, MO, USA)	100 U/mL
				dispase II (Sigma, St. Louis, MO, USA)	0.1%					streptomycin (Sigma–Aldrich, St. Louis, MO, USA)	100 mg/mL
Wang 2010	15-25 y			collagenase type I (Worthington Biochem, Freehold, NJ)	3 mg/mL	60 min	40 µm cell strainer (Falcon, BD, Franklin Lakes, NJ)		DMEM (Gibco-BRL Life Technologies Guangzhou Genewindows Biotech Ltd, Guangzhou, China)	FBS (Gibco-BRL Life Technologies)	20%
										L-glutamine(Gibco-BRL Life Technologies)	
										glucose (Gibco-BRL Life Technologies)	1 g/L
				dispase (Gibco-BRL; Life Technologies, Breda, The Netherlands)	4 mg/mL					penicillin-G (Sigma, St Louis, MO)	100 U/mL
										streptomycin (Sigma, St Louis, MO)	100 µg/mL
Weszl 2012	18-26 y			collagenase type I	3 mg/mL	60 min	70 µm strainer		α-MEM	FCS	20%
										L-ascorbic acid 2-phosphate	100 µM
										L-glutamine	2 mM
				dispase	4 mg/mL					penicillin	100 U/mL
										streptomycin	100 µg/mL

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Woods 2009	15-30 y			collagenase type I (Vitacyte, Indianapolis, IN) collagenase type II (Vitacyte, Indianapolis, IN) thermolysin (Vitacyte, Indianapolis, IN)		15-30 min			Mesencult Complete Medium (StemCell Technologies, Vancouver Canada)		
Yan 2010				collagenase type I (Sigma Aldrich, St. Louis, MO)	3 mg/mL	30-60 min	70 mm cell strainer (Falcon, BD Labware, Franklin Lakes, NJ)		α-MEM (Gibco-Invitrogen, Carlsbad, CA)	FBS (Gemini Bio-Products, Inc., Woodland, CA) L-glutamine (Gemini Bio-Products, Inc.) L-ascorbic acid-2-phosphate (Gemini Bio-Products, Inc.) penicillin-G (Gemini Bio-Products, Inc.) streptomycin (Gemini Bio-Products, Inc.) fungizone (Gemini Bio-Products, Inc.)	15% 2 mM 100 µM 100 U/mL 100 mg/mL 0.25 mg/mL
				dispase (Sigma Aldrich, St. Louis, MO)	4 mg/mL						
Yu 2009				collagenase type I (Worthington Biochemical, Lakewookd, NJ)	3 mg/mL	60 min	70 µm strainer		α-MEM (Gibco Invitrogen)	FBS (Equitech Bio, Kerville, TX) penicillin streptomycin sulfate glutamine	20% 100 U/mL 100 mg/mL 2 mM
				dispase II (Roche Applied Science, Indianapolis IN)	4 mg/mL						
Zhai 2013				collagenase type I (Sigma-Aldrich,St.Louis, MO, USA)	3 mg/mL	45-60 min	70 µm strainer		α-MEM (Hyclone, Logan,UT,USA)	FBS (Hyclone) streptomycin (Roche) penicillin-G (Roche,Basel, Switzerland)	10% 100 mg/mL 100 U/mL
Zhang 2011	16 y	Female		collagenase type I	0.3 mg/mL		40 µm cell sieve	Minced	DMEM/F12	FBS glutaMAX ascorbic acid penicillin streptomycin amphotericin	10% 1% 50 µg/mL 1% 1% 1%
				dispase	0.4 mg/mL						
Zhang 2010	16 y	Male		collagenase type I	0.3 mg/ml		40 µm cell sieve	Minced	DMEM/F12	FBS glutaMAX ascorbic acid penicillin streptomycin amphotericin	10% 1% 50 µg/mL 1% 1% 1%
				dispase	0.4 mg/mL						

Author and year	Age	Gender	Isolation techniques						Culture Medium	Medium supplementation	
			Explant	Enzymatic				Mechanical		Components	Concentration
				Types	Concentration	Time	Filtering				
Zhang 2008 (a)	22 y	Male		collagenase type I	3 mg/mL				α-MEM (Gibco BRL, Life Technologies B. V. Breda, The Netherlands)	FCS (Gibco BRL)	20%
				dispase	4 mg/mL					gentamycin (Gibco BRL)	50 µg/mL
Zhang 2008 (b)	22 y	Male		collagenase type I	3 mg/mL			Minced	α-MEM (Gibco BRL, Life Technologies B. V. Breda, The Netherlands)	FCS (Gibco BRL)	20%
										gentamycin (Gibco BRL)	50 µg/mL
Zhang 2006	18-24 y			collagenase type I	3 mg/mL	60 min	100 µm strainer	Minced	α-MEM (Gibco BRL, Life Technologies B.V. Breda, The Netherlands)	FCS (Gibco BRL)	20%
Zhao 2011	18-35 y	Male		collagenase type I (Sigma, St Louis, MO)	3 mg/mL	60 min	70 µm strainer (Falcon; BD Labware, Franklin Lakes, NJ)		DMEM (Gibco-BRL)	FBS (Gibco-BRL, Grand Island, NY)	15%
										penicillin G (Gibco-BRL)	100 U/mL
		Female		dispase (Gibco-BRL, Grand Island, NY)	4 mg/mL					streptomycin (Gibco-BRL)	100 µg/mL
Zhao 2006				collagenase	625 U/mL				DMEM (Gibco, USA)	FBS (Gibco, USA)	10%
Zhou 2014	18-30 y		Small fragments						DMEM (Life Technologies Pty Ltd., Australia)	FBS (In Vitro Technologies, Australia)	10%
										penicillin (Life Technologies Pty Ltd., Australia)	1%
										streptomycin (Life Technologies Pty Ltd., Australia)	1%

5 **Artigo 2**

How has tooth manipulation been conducted for DPSCs isolation? A systematic review

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Abstract:

The aim of this study was to conduct a systematic review of the literature to obtain the profile of tooth manipulation prior to DPSCs isolation and analyze the possible risk factors that could change the native behavior of these cells. The electronic search was conducted without initial date restriction up to and including (April 2014) in PubMed, Scopus, Scielo and ISI Web of Knowledge databases 222 articles were included and the information analysis was performed concerning the following items: author's name, year of publication, age of donor, sex of donor, tooth storage time between the extraction and DPSCs isolation, tooth storage methods between the extraction and pulp processing, tooth surface cleaning method, dental section method, location of the tooth section and method for removing the pulp from the chamber.. In general, the tooth type most used was the third molar and donor had average minimum of 17.5 y and average maximum equal 30.6 y. The dental storage method was culture medium, and the isolation of DPSCs was performed inmediately after tooth extraction. Many authors neglect dental surface cleaning, however when performed, chlorhexidine was the option of choice. Dental section was carried out predominantly with the fissure bur in the cement-enamel junction. In conclusion, over the past 15 years many studies have been conducted using DPSCs. However, it was observed that there is a clear lack of standardization in tooth manipulation prior to DPSCs isolation. Thus, given the large number of variables aspects in cell isolation technique and all possible consequences in the in vitro behavior of cells, it is important to reinforce the need for standard protocols in order to obtain a more uniform cell culture.

Keywords: Dental pulp stem cell; Manipulation; Dental pulp; Stem cells.

Introduction

Currently, mesenchymal stem cells (MSCs) have been considered a promising alternative for the treatment of a large scale of diseases such as diabetes type 1 (Liu, Cao et al. 2013), liver chronic diseases (Kim, Eom et al. 2015), heart disease (Padda, Sequiera et al. 2015), among others. Although it is recognized that the advances related to the treatment of these diseases are closely associated with the growing number of stem cell research, these studies are mostly developed in animal models, suggesting the need for more research in order to translate the results obtained in animals for humans (de Souza, Alves et al. 2013).

In this context, in order to provide more advances in alternative treatments for human health, it is important to acquire a cell source easily obtainable, with rapid expansion *in vitro* and high proliferative rates. Thus, the dental pulp stem cells (DPSCs) (Gronthos, Mankani et al. 2000) is considered a cell type often eligible for such purposes.

It Is known that DPSCs are in small quantities in the human dental pulp (Fischbach and Fischbach 2004), and therefore it is unquestionable the need for ensuring the best possible way to recreate the cellular microenvironment, in order to achieve sufficient proliferation rate and cell quantity to be used for *in vitro* or *in vivo* purposes (Caplan 2009). However, it is known that teeth manipulation and pulp tissue processing for cell isolation is a complex task to be performed and can be determinant in the success of stem cell isolation (Kerkis and Caplan 2012).

Although dental pulp stem cells have been highly used in studies implicated in clinical applications, the literature is quite scarce considering protocols and discussion of DPSCs isolation techniques and tooth preparation prior to DPSC isolation. Thus, the aim of this study was to conduct a systematic review of the literature to obtain the profile of tooth manipulation prior to DPSCs isolation and analyze the possible risk factors that could change the native behavior of these cells. To the best of our knowledge this is the first systematic review of the literature that evaluated tooth manipulation for DPSCs isolation.

Methods

- Review questions

In the literature, how as tooth manipulation been conducted for DPSCs isolation?

Is there a standardized protocol for tooth manipulation for DPSC isolation?

- Inclusion and exclusion criteria

The inclusion criteria for article assessment were: description of stem cell isolation from human dental pulp and permanent teeth. Exclusion criteria were: literature reviews, book chapters, editorials, letters to the editor, hypothesis articles, news, patents and abstracts sent to congress, articles which are not written in the English language and articles that were not fully available and/or after e-mails sent to the authors there was no reply.

- Search strategy

The electronic search was conducted without initial date restriction up to and including (April 2014) in PubMed, Scopus, Scielo and ISI Web of Knowledge databases in order to identify studies that demonstrate the methodology used for DPSCs isolation. An initial search was conducted using the following MeSH: “(dental pulp stem cell [MeSH])”; “(dental pulp [MeSH])” AND “(stem cell [MeSH])”; “(“dental pulp stem cell” [MeSH]”)”. No language and date restrictions were applied in any search.

All references were managed in the EndNote X7 software (Thomson Reuters, New York, NY, US). Initially, duplicate references were excluded. Titles, abstracts and studies methodologies were screened based on the inclusion and exclusion criteria by two reviewers independently (CPF and EGZC). Lists were compared and in case of disagreement, a consensus was reached by discussion. When a consensus was not achieved, a third reviewer decided if the article should be included (FN). This systematic review followed the PRISMA statements (Moher, Liberati et al. 2009) with some adjustments (Figure 1).

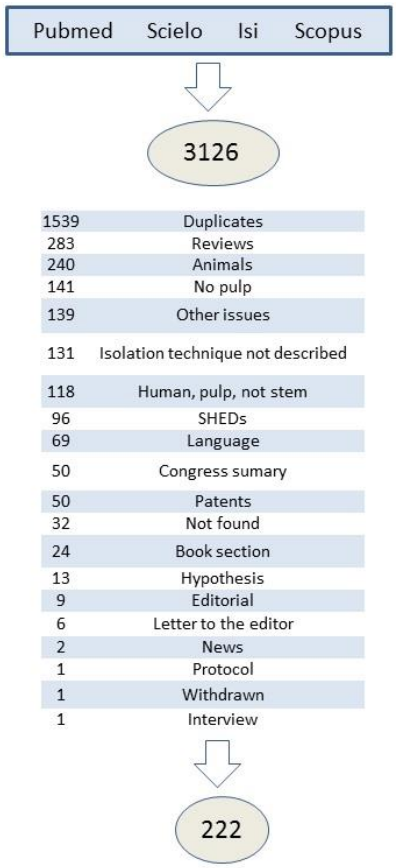


Figure 1 – Flowchart with the studies selection process for inclusion in the systematic review (Exclusion reasons: a study could have fulfilled more than one criterion).

- Data extraction and quality assessment

After screening, the following data were collected from articles that were included in the review: author's name, year of publication, age of donor, sex of donor, tooth storage time between the extraction and DPSCs isolation, tooth storage methods between the extraction and pulp processing, tooth surface cleaning method, dental section method, location of the tooth section and method for removing the pulp from the chamber. Authors were contacted in order to clarify any queries on the study methodology or result. Data were extracted and tabulated by two reviewers (CPF and LCdaR) to be submitted to a descriptive analysis. Cases of disagreement were discussed until a consensus was reached. When a consensus was not obtained, a third reviewer participated in the discussion (FN).

- Assessment of risk of bias in included studies

Risk of bias was evaluated according to the articles description of the following parameters for study quality assessment: (a) donor age, (b) donor gender, (c) tooth type, (d) tooth storage methods, (e) tooth storage time, (f) dental surface cleaning (g) dental section methods and (h) pulp removal methods. If the authors reported the parameters, the article had a “Y” (yes) on that specific parameter; if it had the parameter, however it was not specific, the article was marked with an “U” (unclear); and finally in the information was not described, the article received a “N” (no). After the evaluation the data were exported into Review Manager 5.3 for analysis and generate graph.

- Impact factor analysis

The impact factor analysis, of the included articles, was performed searching each journal for its impact factor in the year that the corresponding study was published. Then these values were categorized as follows: From 0 to 1, more than 1 to 2, more than 2 to 3, more than 3 to 4, more than 4 to 5, more than 5 to 6, more than 6 to 7, more than 7 to 8, more than 8 to 9, more than 9 up to 10, more than 10. Subsequently, a relation between the impact factor and the presence or absence of the following items was established - a) age of donor, b) gender of donor c) tooth storage time, between the extraction and DPSCs isolation, d) tooth storage methods, between the extraction and pulp processing, e) tooth surface cleaning method, f) dental section method, g) location of the tooth section and h) method for removing the pulp from the chamber.

Results

- Descriptive Analysis

Database search revealed 3126 articles. From those 1539 were duplicated and therefore were removed. A total of 1587 articles were included for title, abstract and methodology evaluation. Figure 1 shows flow-chart of the study selection. From those 222 were included for full text analysis. Tooth donor profile is predominantly males (55.9%) with an average age equal to 17:5 years and average maximum age equal to 30.6 years.

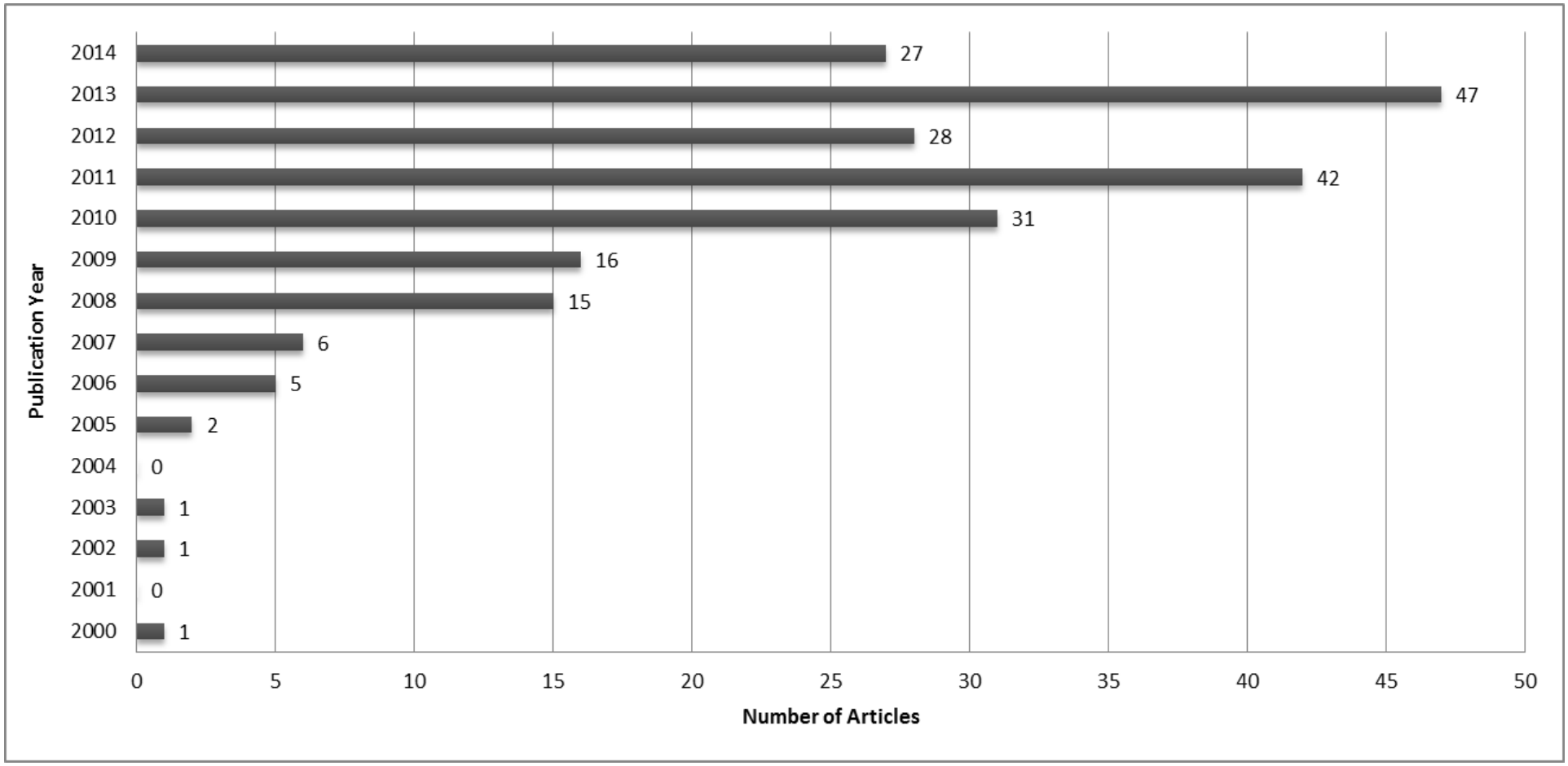


Figure 2 – Distribution of articles included in the systematic review according to the year of publication.

From the studies included in this review, 84.2% described the tooth type used for DPSCs isolation. The second item most frequently mentioned by authors was the donor age, representing 67.6% of total studies. However among the aspects analyzed, the tooth storage time, between the extraction and DPSCs isolation, was the most neglected information not being informed in 88.3% of the articles. Followed by the lack of data on tooth storage method used between the extraction and the initiation of DPSCs isolation procedure, corresponding for 84.7% (Table 1).

	Yes		No	
	N	%	N	%
Tooth type	187	84.2%	35	15.8%
Donor age	150	67.6%	72	32.4%
Donor gender	35	15.8%	187	84.2%
Tooth storage methods	34	15.3%	188	84.7%
Tooth storage time	26	11.7 %	196	88.3%
Dental surface cleaning	85	38.3%	137	61.7%
Dental section method	84	37.8%	138	62.2%
Dental section site	42	18.9%	180	81.1%
Pulp removal methods	54	24.3%	168	75.7%

Table 1. Distribution according to the presence or absence of important aspects of DPSCs isolation.

Therefore, the vast majority of studies mentioned the type of tooth used for DPSCs isolation (Table 1). In this context, data shows that the third molar is the most commonly type of teeth used, representing more than 77.0%. However, it is suggested that this number is even higher, since 9.2% of the total are molars without type specification - first, second or third molar. The second group of teeth most commonly used is premolar, representing approximately 10.7%. Supernumerary and incisors teeth represent 2.6% and the use of canine teeth was not mentioned in any study (Table 2).

	N	%
Supernumerary		
	2	1.0%
Incisor		
Incisor (not described the type)	1	0.5%
Left upper central incisor - traumatized	1	0.5%
Left upper lateral incisor - traumatized	1	0.5%
Incisor total	3	1.5**%**
Premolar		

Premolar (not described the type)	18	9.2%
First premolar	2	1.0%
Lower premolar	1	0.5%
Premolar total	21	10.7%
Molar		
Molar (not described the type)	18	9.2%
Third molar		
Third molar (not described the type)	88	44.9%
Impacted third molar	57	29.1%
Upper third molar	3	1.5%
Mandibular third molar	1	0.5%
Non-erupted third molar	1	0.5%
Semi-erupted third molar	1	0.5%
Semi-impacted third molar	1	0.5%
Third molar total	152	77.6%
Total	196	100.0%

Table 2. Distribution according to the types of teeth used to isolate DPCSs.

In relation to the storage method used for transportation of the newly extracted teeth to the site of DPSCs isolation, only 15.3% mention the storage method used (Table 1). Seven different types of solutions were cited. Furthermore, 5.9% of total studies mention the use of a transportation solution but do not describe the type used (Table 3). The most common material used is the culture medium, present in over 44.1% of the studies, followed by HBBS, present in 20.6% of the articles (Table 3). Then, not least, there is the time spent between the extraction and isolation. However, over 88.0% of the articles do not report the time spent between these two steps. The majority of authors, 55.6% recommend the isolation of DPSCs immediately after extraction of dental elements and over 92.6% opting for isolation within 24 hours (Table 4).

	N	%
Culture medium	15	44.1%
HBBS	7	20.6%
PBS	4	11.8%
DPBS	2	5.9%
Saline solution	2	5.9%
Freezing medium	1	2.9%
FBS	1	2.9%
Stored (not described)	2	5.9%
Total	34	100.0%

Table 3. Distribution according to the storage solution used for transportation of the newly extracted teeth to the site of DPSCs isolation. Abbreviations: Hank's buffered salt solution - HBBS; phosphate buffered saline –PBS; Dulbecco's phosphate buffered saline – DPBS; Fetal bovine serum – FBS.

	N	%
Immediately	15	55.6%
Within 2 h	6	22.2%
Within 24 h	4	14.8%
6 to 48 h	1	3.70%
72 h	1	3.70%
Total	27	100.0%

Table 4. Distribution according to the tooth storage time, between the extraction and DPSCs isolation.

Around 38% of authors reported executing some method of tooth surface cleaning before sectioning the dental element for access to the pulp chamber (Table 1). In all, 13 different types of tooth surface cleaning solutions are mentioned which are used alone or in combination with each other. 24.1% of the studies mention realizing the teeth surface cleaning, however did not describe how it was done. PBS and CHX were the most used solutions, each present in 14.7% of the studies. However, the CHX is presented in various ways, by varying the concentration used between 0.2 and 0.3%, as well as the form of administration, solution or gel (Table 5).

	N	%
CHX		
0,2%	3	2.6%
0,2% solution	1	0.9%
0.3%	3	2.6%
0,3% solution	7	6.0%
0,3% gel	7	6.0%
Not described the type	2	1,69%
Solution	1	0.9%
CHX Total	17	14.7%
PBS	17	14.7%
Professional Hygiene	15	12.9%
DPBS	9	7.8%
Ethanol	9	7.8%
PVP-I	8	6.9%
Physiological solution	4	3.5%
Destilled water	3	2.6%

Sodium thiosulfate	2	1.7%
Sterile surgical blade	1	0.9%
HBSS	1	0.9%
Dental burs	1	0.9%
Dental scaler	1	0.9%
Not described	28	24.1%
Total	116	100.0%

Table 5. Distribution according to the tooth surface cleaning method. Abbreviations: clorhexidina - CHX; phosphate buffered saline - PBS; Dulbecco's Phosphate-Buffered Saline - DPBS; povidone-iodine - PVP-I; Hank's Balanced Salt Solution - HBSS.

After cleaning the tooth surface, the teeth are sectioned so that the pulp chamber and finally the pulp tissue can be access. However, only 37.8% of the papers report the section step (Table 1). Among those, the most used tools described are the fissure bur, forceps and diamond discs, representing 24.7%, 18.3% and 11.8% respectively. Another 19 methods to perform dental section are mentioned and represent 36.56% of the sample, these data was grouped since individually each one was unrepresentative (Table 6).

	N	%
Fissure bur	23	24.7%
Forceps	17	18.3%
Diamond discs	11	11.8%
High speed	8	8.6%
Other 19 methods	34	36.6%
Total	93	100.0%

Table 6. Distribution according to method dental section.

Few studies explain the location where the teeth was sectioned in order to have access to the pulp chamber and the dental pulp tissue, Almost all studies (88.1%) chose to perform the dental section at the cementum enamel junction (CEJ) (Table 7).

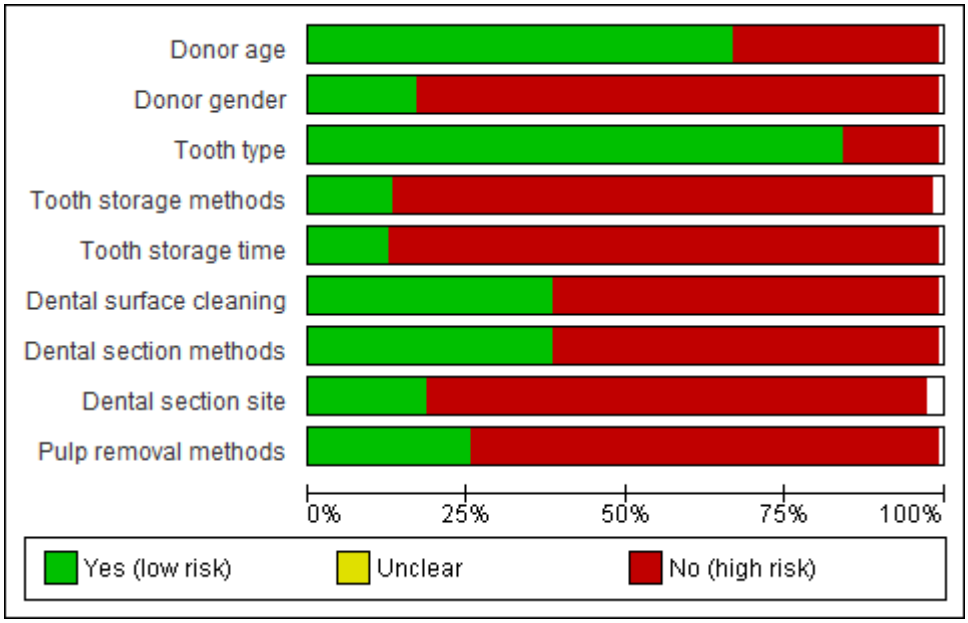
	N	%
CEJ	37	88.1%
Root enamel boundary	4	9.5%
Crown-root border	1	2.4%
Total	42	100.0%

Table 7. Distribution according to location of the dental section. Abbreviation: Cementum enamel junction – CEJ.

In relation to the methods used to remove the pulp tissue inside the dental chamber, 75.7% of the authors do not mention this step in the article (Table 1). Eight different instruments are cited to remove the pulp tissue. The most commonly used instrument is the excavator, present in 59.7% of the articles. The second most used instrument is the forceps, representing 11.7% of the total studies (Table 6).

- Risk of Bias

Of the 222 studies included, only two variables – age and tooth type, more than 50.0% of article showing low risk of bias. When analyzing the risk of bias over the variable gender of donor, it is observed that less than 25.0% of the articles had low risk of bias. The majority of studies, more than 75.0%, no report the tooth storage time, tooth storage method and method for removing the pulp from the chamber, representing high risk of bias. About the risk of bias for tooth surface cleaning method and local dental section, between 25.0 and 50.0% of studies representing low risk of bias (Graph 1).



Graph 1. Risk of bias considering aspects reported in the material and method section

- Impact factor

The analysis between the impact factor and the presence or absence of fundamental aspects in dental manipulation for DPSCs isolation is shown in table 8. A higher relative frequency is observed in relation to the donors age, type of tooth, storage medium and storage time in articles published in journals with impact factor from 0 to 5, in comparison to journals with impact factor ranging from 5 to higher than 10. On the other hand information's related to gender, tooth surface cleaning method, dental section method, location of tooth section and method for removing the pulp tissue from the chamber had a higher relative frequency in journal with impact factor ranging from 5 to higher than 10.

Impact Factor	Age % (n)		Gender % (n)		Tooth type % (n)		Storage medium % (n)		Storage time % (n)		Tooth surface cleaning method % (n)		Dental section method % (n)		Location of tooth section % (n)		Method for removing the pulp from the chamber % (n)	
	Y	N	Y	N	Y	N	Y	N	Y	N	Y	N	Y	N	Y	N	Y	N
0 to 1	100 (17)	0.0 (0)	29.4 (5)	70.6 (12)	100 (17)	0.0 (0)	37.5 (6)	62.5 (10)	35.3 (6)	64.7 (11)	35.3 (6)	64.7 (11)	47.1 (8)	52.9 (9)	11.8 (2)	88.2 (15)	58.8 (10)	41.2 (7)
1 to 2	100 (37)	0.0 (0)	18.9 (7)	81.1 (30)	100 (37)	0.0 (0)	16.2 (6)	83.8 (31)	2.7 (1)	97.3 (36)	45.9 (17)	54.1 (20)	37.8 (14)	62.2 (23)	18.9 (7)	81.1 (30)	16.2 (6)	83.8 (31)
2 to 3	100 (49)	0.0 (0)	10.2 (5)	89.8 (44)	100 (49)	0.0 (0)	10.2 (5)	89.8 (44)	12.2 (6)	87.8 (43)	26.5 (13)	73.5 (36)	36.7 (18)	63.3 (31)	22.4 (11)	77.6 (38)	26.5 (13)	73.5 (36)
3 to 4	100 (46)	0.0 (0)	17.4 (8)	82.6 (38)	100 (46)	0.0 (0)	15.2 (7)	84.8 (39)	15.2 (7)	84.8 (39)	43.5 (20)	56.5 (26)	37 (17)	63.0 (29)	13 (6)	87 (40)	10.9 (5)	89.1 (41)
4 to 5	4.8 (1)	95.2 (20)	9.5 (2)	90.5 (19)	100 (21)	0.0 (0)	9.5 (2)	90.5 (19)	0.0 (0)	100 (21)	28.6 (6)	71.4 (15)	14.3 (3)	85.7 (18)	9.5 (2)	90.5 (19)	19 (4)	81 (17)
5 to 6	0.0 (0)	100 (6)	50 (3)	50 (3)	100 (6)	0.0 (0)	0.0 (0)	100 (6)	16.7 (1)	83.3 (5)	66.7 (6)	33.3 (2)	83.3 (5)	16.7 (1)	50 (3)	50 (3)	50 (3)	50 (3)
6 to 7	0.0 (0)	100 (6)	0.0 (0)	100 (6)	100 (6)	0.0 (0)	0.0 (0)	100 (6)	0.0 (0)	100 (6)	66.7 (4)	33.3 (2)	50 (3)	50 (3)	50 (3)	50 (3)	16.7 (1)	83.3 (5)
7 to 8	0.0 (0)	100 (8)	12.5 (1)	87.5 (7)	62.5 (5)	37.5 (3)	12.5 (1)	87.5 (7)	12.5 (1)	87.5 (7)	37.5 (3)	62.5 (5)	50 (4)	50 (4)	12.5 (1)	87.5 (7)	25 (2)	75 (6)
8 to 9	0.0 (0)	100 (2)	50 (1)	50 (1)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	50 (1)	50 (1)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (2)

9 to 10	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	0.0 (0)	100 (2)	50 (1)	50 (1)	50 (1)	50 (1)	0.0 (0)	100 (2)
Higher then 10	0.0 (0)	100 (3)	0.0 (0)	100 (3)	0.0 (0)	100 (3)	0.0 (0)	100 (3)	0.0 (0)	100 (3)	33.3 (1)	66.7 (2)	66.7 (2)	33.3 (1)	66.7 (2)	33.3 (1)	33.3 (1)	66.7 (2)
Not found	0.0 (0)	100 (25)	12 (3)	88 (22)	0.0 (0)	100 (25)	28 (7)	72 (18)	16 (4)	84 (21)	40 (10)	60 (15)	36 (9)	64 (16)	16 (4)	84 (21)	36 (9)	64 (16)
Total lower then 5	88.2 (150)	11.8 (20)	15.9 (27)	84.1 (143)	100 (170)	0.0 (0)	15.4 (26)	84.6 (143)	11.8 (20)	88.2 (150)	36.5 (62)	63.5 (108)	35.3 (60)	64.7 (110)	16.5 (28)	83.5 (142)	22.4 (38)	77.7 (132)
Total higher then 5	0.0 (0)	100 (27)	18.5 (5)	81.5 (22)	63 (17)	37 (10)	3.7 (1)	96.3 (26)	7.4 (2)	92.6 (25)	51.7 (15)	48.3 (14)	55.6 (15)	44.4 (12)	37 (10)	64 (17)	25.9 (7)	74.1 (20)

Table 8. Relation of the impact factor of the journals in the years of publications articles with the presence of some variables of interest.

Discussion

This systematic review demonstrates that DPSCs isolation has been investigated over the past 14 years, since it was first reported in 2000. In the first five years following the discovery of DPSCs, only 5 articles were published. The publication frequency raised in 2006 with 5 publication, and continue to increase in the following years reaching its peak in 2013. This reflects that while the interest of researchers for DPSCs was immediate, the low number of publications over the first years was due to the time required for study development in this area, and therefore enhanced its number in the past year.

According to Gronthos et. al, DPSCs derived from molar teeth have a greater degree of cell proliferation *in vitro*, when compared with bone marrow stem cells (BMSCs), and this behavior is attributed to differences in the development stage of each organ (Gronthos, Mankani et al. 2000). Within this context, as Table 2 indicates, the teeth most commonly used to obtain DPSCs are third molars, which represent over 77% of choice of all authors (Table 2). The preference for this particularly group of teeth can be explained since they would be the last ones to complete the development stages (Gronthos, Mankani et al. 2000). It is know that in adolescents and young adults, the third molars are the only teeth still developing (Bhat and Kamath 2007). In addition, third molars raise interest since they are easily accessible and are commonly indicated for extraction for orthodontic reasons, usually between the ages of 16 and 18 (Kellner, Steindorff et al. 2014). Nevertheless, the literature has suggested that DPSCs derived from third molars have the same expression profile gene of induced pluripotent stem cells (IPSCs) (Atari, Barajas et al. 2011). IPSCs are somatic cell transformed by the integration in the genome of a set of transcriptions factors which convert their status to a pluripotent state, in similarity to embryonic stem cells,. Thus, these cells seems interesting because, they are similar the stem cells as embryonic morphology, proliferation and formation of teratomas, yet they are different with respect to gene expression and patterns DNA methylation (Okita, Ichisaka et al. 2007). Furthermore, they are an alternative to circumvent ethical concerns about using cells of embryonic origin and problems related to immune system (Takahashi and Yamanaka 2006).

Our findings suggest that most studies neglects the report on the patients gender, accounting for over 84.2% of all studies included in this review. Among the studies that report the sex of the donor (15.8%), the most prevalent individuals are male,

accounting for 55.9% (Table 1). It has been reported that in women's the final development of the upper and lower third molar occurs averagely at 22.3 and 21.6 years, respectively. In men, the upper and lower third molar complete their development at approximately 22.4 and 21.8 years, respectively (Jafari, Mohebbi et al. 2012). In relation to the donor's age, our finding shows that 67.6% mentioned this variable (Table 1), suggesting that literature has a low risk of bias (Figure 1). Nevertheless, the donors had a minimum and maximum age average of 17.5 and 30.6 years, respectively. It is well known that stem cells are required for continuous supply of mature and functionally competent cell for normal tissue turnover and regeneration (Nedel, Andre Dde et al. 2009). Age-related issues could decrease cell number or function leading, therefore, to tissue deterioration (Hartwig, Nedel et al. 2012). It has been suggested that stem cells from the marrow from younger individual could have a greater pool size. This would be related to the changes in the skeletal dynamics from a modeling mode characteristic of the skeletal growth and consolidation to a remodeling dynamic characteristic of the adult skeleton (Bellantuono, Aldahmash et al. 2009). The same correlation could be thought for DPSCs, were the most indicated moment for stem cell isolation would be in the teeth final development, were stem cell are still in the growth and consolidation dynamics, rather then in the remodeling phase. In addition, recently, a study on cell growth showed that cells from individual with less than 22 years have lower doubling time than DPSCsfrom donors with 22 years or more. However, although the donor's age plays an important role in cell doubling time, the individual differences of each donor seems to be more important than age-dependent differences (Kellner, Steindorff et al. 2014). These results can be associated with stem cells telomere shortening and increase sings associated with senescence.

Based on the importance of acquiring the maximum cell population for a single tooth, and therefore obtaining a lower doubling cell time, it is highly recommended that authors use teeth in their minimal state of development; acquiring, therefore, upper and lower third molars in the range of 22.3 and 21.6 years, respectively, for females and 22.4 and 21.8 years, respectively, for mans.

Many times teeth extraction and dental pulp tissue processing occurs in different locations, thus, the teeth transportation time and conditions, becomes an important issue (Perry, Zhou et al. 2008, Ducret, Fabre et al. 2015). However, our results indicate that the literature neglects massively these two aspects (Table 1). Even

though the need for teeth conditioning, that does not affect cell viability, is evident (Perry, Zhou et al. 2008), our results show more than 83% of the articles with high risk of bias for this variable (Figure 1). Among the studies reporting the teeth storage condition, seven different storage methods were described. The most preconized ones were: the same culture medium used subsequently the DPSCs, Hank's balanced salt solution and PBS, representing respectively 44.1%, 20.69% and 11.7% of total included studies.

In relation to the dental elements storage time, this review shows that over 88.3% of the studies do not report this information (Table 1). Among the studies that report the teeth storage time, 92.6% prefer to initiate cell dissociation within 24 hours after the tooth extraction. It was believed that DPSCs would be viable only when isolated within 48 hours after dental extraction. On the contrary, Perry et al. in 2008 suggested that DPSCs when maintained in cell culture medium, PBS or Hypo Thermosol (HTS) can be conserved viable for up to 120 hours (Perry, Zhou et al. 2008).

Another important issue considering teeth processing prior to DPSCs isolation is the cleaning procedure of the dental surface. Our review showed that nearly 61.7% of all studies do not mention the process used in dental surface cleaning prior to dental section in order to access the pulp tissue (Table 1). However, this procedure could represent a critical step to achieve a lower risk of cell culture contamination, since more than 700 bacterial species or phylotypes (Aas, Paster et al. 2005), mycoplasmas (Kim, Kim et al. 2015) and a wide range of fungi, especially *Candida* and *Streptococcus* species (Xu and Dongari-Bagtzoglou 2015), are detected in the oral cavity. The authors who have chosen to perform this step suggest, mainly the use of substances such as chlorhexidine, representing almost 15% of the total study. Chlorxidine has been shown to present an antibacterial and antifungal properties (Thomas, Thakur et al. 2015), and therfore justifing its use for dental surface cleaning prior to dental section and DPSCs isolation. Chlorhexidine performs bactericidal activity in high concentrations and bacteriostatic at low concentrations (Asokan, Emmadi et al. 2009). This could explain why the 3.5% of the authors opted for disinfecting the teeth surface with chlorhexidine in a concentration of 0.2%, while 8.6% of the authors used a higer concentration, 0.3% of chlorhexidine. Another straind of researchers used mechanical tools for dental surface cleaning such as dental burs, dental scaler and sterile surgical blade, present in 2.6% of the studies.

There are also those who suggest profissional profilaxy of the oral cavity prior to tooth extraction, representing 12.9%.

Since Gronthos et al., in 2000, was the pioneer in isolating DPSCs, they provide the basis for many researchers. In agreement to the methodology mentioned by Gronthos (Gronthos, Mankani et al. 2000), among the authors who report the section site of the dental, structure to access the pulp chamber, 88.1% sectioned the tooth structure around the cementum enamel junction and 24.7% used the dental fissure burs. However, in order to perform this step of the methodology it is required the presence of a high speed handpiece coupled to a dental unit in the laboratory context, which in fact, is unlikely to exist. Thus, performing this step of the methodology commonly occurs in installations where dental clinical procedures are performed, hindering the maintenance of a sterile environment to prevent contamination of the pulp tissue, and consequently the cell culture. Subsequently, once the pulp chamber has been accessed, the next step is to remove the pulp tissue. From all the articles included in this review 75.7% of the authors do not mention this step. According to our findings, the use of excavators is the choice of approximately 60% of authors who mention any methods of dental pulp tissue removal.

In recent years, our research group has been performing the isolation of dental pulp stem cells. Thus, the dental opening methodology proposed consists in realize two groove, one at the occlusal surface and the other on the proximal surface toward the long axis of the tooth structure. These groove are performed with a diamond bur conical trunk (number 4138) on high-speed handpiece, under constant irrigation milique sterile water. The groove should measure about 2 mm, size considered sufficient to support a chisel in the proximal groove and and through the impact of a dental hammer supported at handle of the chisel, promoting the dental structure fracture line, providing access to the camera and pulp tissue. They are carried out with the aid of a diamond bur conical trunk (number 4138) on high-speed handpiece, under constant irrigation Milique sterile water. The channels should measure about 2 mm, size considered sufficient to support a chisel in the proximal groove and through the impact of a dental hammer supported at handle of the chisel, then, promoting the dental structure fracture line, providing access to the camera and pulp tissue. The literature suggests that thermal damage should be avoided, since pulp tissues undergoing increased intrapulpal temperature of 5.5°C can cause irreversible pulp

damage (Visuri, Walsh et al. 1996). Thus, our methodology seems advantageous over the complete section with high-speed handpiece, once performs shallow groove and with intermittent movements, avoiding impairment of the cell viability.

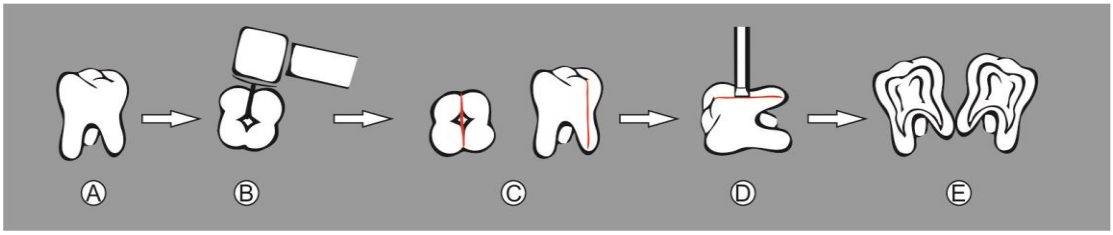


Figure 3 – Schematic method suggested for dental section and access to the pulp chamber. (A) Cleaning of the tooth surface (B) Groove performed with a diamond bur truncated conical at low speed (C) Groove in the occlusal and proximal faces, towards the long axis of the tooth (D) Support of chisel in the proximal groove and hammered on the chisel (E) Tooth sectioned into two parts with access to pulp chamber.

After obtaining the dental pulp from the pulp chamber, DPSCs can be isolated using two main techniques: explant (Kerkis, Kerkis et al. 2006, Karamzadeh, Eslaminejad et al. 2012, Lizier, Kerkis et al. 2012) and enzymatic (Kerkis, Kerkis et al. 2006, Karamzadeh, Eslaminejad et al. 2012). It is also recurrent the association between the enzymatic technique and the use of mechanical devices to intensify cell dissociation from each other and the extracellular matrix (Patil, Kumar et al. 2014).
The analysis between the impact factor and the presence or absence of fundamental aspects in dental manipulation for DPSCs isolation shows a higher relative frequency in relation to the donors age, type of tooth, storage medium and storage time in articles published in journals with impact factor from 0 to 5. On the other hand information's related to gender, tooth surface cleaning method, dental section method, location of tooth section and method for removing the pulp tissue from the chamber had a higher relative frequency in journal with impact factor higher than 5. However this systematic review shows clearly the lack of information in journal, independent of the impact factor, since a high risk of bias was found in almost all variables evaluated, with exception to donors age (67.6%) and tooth type (84.2%). We evaluate that information such as donors age, type of tooth, storage medium, storage time, tooth surface cleaning method, dental section method, location of tooth section and method for removing the pulp tissue from the chamber should be

standardized information present in all original articles, so that future protocols could be well established.

Based on the frequency in each step of tooth manipulation for DPSCs isolation, obtained in our systematic review, we developed a standardized protocol (Figure 2). However it is important to highlight that this protocol is based on frequency analysis and not in effectiveness of tooth manipulation.

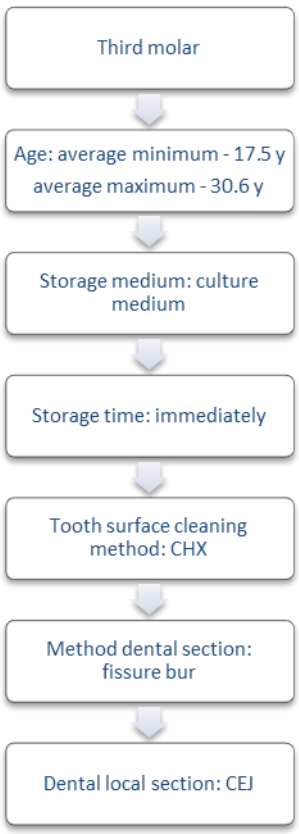


Figure 4 – Flowchart with the most commonly used protocol for tooth manipulation to conduct to DPSCs isolation.

Conclusion

Over the past 15 years many studies have been conducted using DPSCs. However, it was observed that there is a clear lack of standardization in tooth manipulation prior to DPSCs isolation. Thus, given the large number of variables aspects in cell isolation technique and all possible consequences in the in vitro behavior of cells, it is important to reinforce the need for standard protocols in order to obtain a more uniform cell culture.

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Supplementary material

Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
	DPSCs	10-40 y			Within 24 h	Immerssion in 75% ethanol and soaking in PBS		
Third molar	DPSCs	24–35 y			2 h	Root surface cleaned with PVP-I (Sigma-Aldrich, St. Louis, MO, USA)		
Impacted third molar Semi-impacted third molar	DPSCs	18–28 y				Rinse mouth with CHX before extraction	Cow horn forceps	Small escavator
Third molar	DPSCs	20-29 y		DMEM (Biowhittaker, Gibco, Sigma, USA), penicillin/streptoycin (Invitrogen Co, USA) and 10% FBS (JRH biosciences, Inc., Lenexa, KS, USA)				Sterile dental probe
Third molar	DPSCs	18–25 y		PBS contaning antibitics, on ice			Cut around the circumference of the teeth using a sterile hand-held high-speed drill at the CEJ level	Endodontic file
Impacted third molar	hDPSCs	16–24 y		Cell culture medium				
Impacted third molar	DPSCs-NPs DPSCs-IPs	14-22 y		Tissue freezing medium (Triangle Biomedical Sciences, Durham, NC, USA) or				
Third molar	DPSCs	18-21 y						
Impacted third molar	DPSCs	19-35 y				Cleaned (not described)	Torno (vice)	
Third molar	DPSCs					Disinfected by 70% ethanol	Dissected at the crown-root border	
Third molar	DPPSCs DPMSCs	18–27 y	Male Female		Immediatly	Washed using gauze soaked in 70% ethanol, followed by a wash with sterile destilled water	Hold the tooth with upper incisor fórceps. The incision was in JEC by using a cylindrical turbine bur	Sterile nerve-puller file 15 and forceps
Third molar	DPPSCs DPMSCs	14–60 y	Male Female		Immediatly	Washed using gauze soaked in 70% ethanol, followed by a wash with sterile destilled water	Hold the tooth with upper incisor fórceps. The incision was in JEC by using a cylindrical turbine bur	Sterile nerve-puller file 15 and forceps
Third molar	DPMSCs	14-60 y	Male Female		Immediatly	Washed using gauze soaked in 70% ethanol, followed by a wash with sterile destilled water	Hold the tooth with upper incisor fórceps. The incision was in CEJ by using a cylindrical turbine bur	Sterile nerve-puller file 15 and forceps

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Attar 2014	Molar	PPSCs						Teeth cut around the root enamel boundary using dental fissure bur	Polyps were removed with curettage
	Third molar	DPSCs							
Bakopoulou 2011	Impacted third molar	DPSCs	16-18 y		.		Cleaned (not described)	Cut around the CEJ	
Batouli 2003	Impacted third molar	DPSCs	19-29 y						
Bonnamain 2013	Non-erupted third molar		15–20 y	Male Female					
Bressan 2012	Molar	DPSCs	16-66 y				Pretreatment for one week with professional dental hygiene. Before extraction dental crown covered with 0.3% CHX gel (Forhans, New York, NY) for 2 min	Mechanical fracturing	Dentinal excavator or a gracey curette
Cai 2011	Impacted third molar	hDPSCs	18-28 y						
Carinci 2008	Molar								Dentinal excavator or a gracey curette
Carvalho 2012	Upper third molar	hDP-DPSCs					Cleaned (not described)	Diamond cutter disc	
Chen 2013		hDPSCs - liquid nitrogen stored dental pulp tissues	Mean of 26.5 y						Endodontic file
		hDPSCs - freshly derived dental pulp tissues	Mean of 23.4 y						
Chen 2012	Third molar	DPSCs	19-23 y		α- MEM serum free (Hyclone,	1 h			
Chen 2011		hDPSCs:	Mean of 25.5 y, 6–74 y	Male Female			Cleaned the tooth surface with DPBS	A circumference groove of 0.5–1.0 mm in depth was cut around the entire tooth using an aseptic high speed handpiece. The tooth was split using a chisel.	Endodontic file
		freshly derived dental pulp tissues	Mean of 26.5 y, 6-74 y						
		liq N2-stored dental pulp tissues	Mean of 23.4 y, 6-49 y						
		liq N2-stored intact teeth	Mean of 27.7 y, 8-52 y						

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Choi 2012	Molar	DPSCs			HBSS (Welgene, Dae-gu, Korea) with 3% AA (Life Technologies, Carlsbad, CA) at 4°C			Dental high-speed unit	
Chun 2011 (a)	Third molar	DPSCs							
Chun 2011 (b)		DPSCs							
Cmielova 2013	Impacted thrird molar	DPSCs	Mean of 19 y, 12-23 y	Male Female	Stored (not described)		Cleaned (not described)		
Collart-Dutilleul 2014	Impacted third molar	DPSCs	15–18 y				Tooth surfaces were cleaned using 0,2% CHX	Cut around the CEJ	
Cui 2014	Third molar	hDPSCs							
Cui 2013	Third molar	hDPSCs							
Dai 2012	Third molar	DPSCs	Mean of 18+-3.2, 16-25 y						
D'Alimonte 2013		DPSCs	Mean of 17 y	Male Female					
D'Alimonte 2011		DPSCs	Mean of 17 y	Male Female					
D'Aquino 2009	Third molar	DPSCs		Male Female			Professional oral hygiene one week before surgery 0,2% CHX after brushing , twice a day	Surgical drill	
D'Aquino 2007		DPSCs		Male Female			Professional oral hygiene one week before surgery 0,3% CHX after brushing , twice a day	Surgical drill	Dentinal excavator or a gracey curette
de Rosa 2011		DPSCs	21-45 y						
de Souza 2010	Impacted third molar	DPCp	9-15 y				Cleaned (not described)	Longitudinal groove and sterilized diamond discs (KGSorensen, ref.7020, Zenith Dental ApS, AgersKov, Denmark)	Sterile dentinal excavator
Demircan 2011	Molar	hDP-SC	20 y	Male			Immerssion in physiologic solution containing antibiotics	Pliers (bone forceps)	

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Diomede 2013	Premolar	hDPSCs	18-25 y				Rinsed five times with PBS containing penicillin and streptomycin	Cylindrical diamond bur (314, Ø ISO 014, L.8.0mm Intensiv, Grancia, Switzerland) mounted on a high speed handpiece (Bora L; Bien Air, Bienne, Switzerland) with water spray cooling	Sterile excavator
Dissanayaka 2012	Third molar	DPSCs	18-25 y				Cleaned (not described)	Sterile fissure bur at the CEJ	
Dissanayaka 2011	Third molar	hDPSCs					Cleaned (not described)	Cut at the CEJ by using a sterile fissure bur	
Djouad 2010	Third molar	DP-MPCs	16-26 y						
Dolatshahi-Pirouz 2010	Third molar	DP-MSC	21 y						
Duailibi 2011	Mandibular third molar	DSCs			HBSS (Gibco BRL, Gaithersburg, MD, USA) pre-warmed 37°C				
Ebrahimi 2011		DPSCs			PBS solution containing penicillin and streptomycin on ice		Disinfected in iodine solution, washed with PBS	Cracked using a turbine along the cervical region and with sterilized cowhorn forceps	Gracey curette
Egbuniwe 2011	Third molar	tDPSCs					Washed with 70% ethanol and then with HBSS (Invitrogen) p.H 7.4	Horizontal indentations along the cervical margin using a low speed circular diamond circular diamond saw (Agar Scientific Ltd.)	Sterile forceps
		nDPSCs							
Eleutério 2013	Premolar teeth	DPSCs	20-35 y				Professional oral hygiene one week before surgery. Cleaned with PBS containing penicillin and streptomycin	Cylindrical diamond rotary cutting instrument m ounted on a high-speed handpiece with water-spray cooling	Sterile dentinal exacavator
Eslaminejad 2013	Third molar	DPSCs	20-25 y	Male				Cut around the root-enamel boundary using dental fissure bur	
Eslaminejad 2010 (a)	Third molar		20-25 y					Cut around the root-enamel boundary using dental fissure bur	
Eslaminejad 2010 (b)	Third molar		20-25 y						
Eslaminejad 2009	Third molar		20-25 y					Cut around the root-enamel boundary using dental fissure bur	
Eubanks 2014		DPSCs	15–22 y		Sterile saline solution or α- MEM containing 15% FBS	Immediately 24 h at 4°C		Cut above the CEJ	

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Fang 2013	Third molar	DPSCs	16-30 y		Culture medium containing 100 U/mL penicillin and 100 mg/mL streptomycin		Desinfected with PVP-I for 5 min PBS containing 100 U/mL penicillin and 100 mg/mL streptomycin		
Feng 2014	Impacted third molar	DPSCs	13-23 y				Cleaned (not described)	Cutting around the CEJ using sterilised dental fissure bur	
Feng 2013 (a)	Impacted third molar	DPSCs	13-23 y				Cleaned (not described)	Cutting around the CEJ using sterilised dental fissure bur	
Feng 2013 (b)	Impacted third molar	DPSCs	45-50 y				Dental pulp tissue was washed three times with PBS		
Foudah 2014	Third molar	hDPSCs			PBS	Maximum of 1h		Piezoelectric ultrasonic device (OT7 insert) under abundant irrigation with sterile 0.9% NaCl	MOD.31W hand excavator
Gabanyi 2013	Third molar						Cleaned (not described)		
Gandia 2008	Third molar	DPSCs	18-21 y						
Gay 2014	Third molar	DPSCs			DMEM containing 10% FBS, 1% penicilin and streptomycin	72 h			
Giorgini 2011	Third molar	DPSCs	18-20 y				Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur	
Govindasamy 2011	Third molar	DPSCs	24 – 35 y		1 X DMEM-KO, 10% FBS, 2% penicillin,2% streptomycin, 5% GlutaMax, 100 mg/mL ascorbic acid, 1X ITS	2 h	Root surfaces were cleaned with 100% PVP-I (Sigma Aldrich, St Louis, MO)		
Govindasamy 2010 (a)	Third molar	DPSCs	24-35 y			2 h	Root surfaces were cleaned with PVP-I (Sigma Aldrich, St Louis, MO)		
Govindasamy 2010 (b)		DPSCs	14–25 y						
Graziano 2008		SBP-DPSC	21–45 y				Petreated for a week with professional dental hygiene. Before extraction, the dental crown crown was covered with a 0,3% CHX (Forhans,New York, NY) for 2 min		Dentinal excavator or gracey curette
Graziano 2007		SBP-DPSC	25-45 y				Petreated for a week with professional dental hygiene. Before extraction, the dental crown crown was covered with a 0,3% CHX (Forhans,New York, NY) for 2 min		Dentinal excavator or gracey curette

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Gronthos 2000	Impacted third molar	DPSCs	19-29 y				Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur	
Han 2008	Third molar	DPSCs					Tooth was washed with PBS containing AA solution for 3 min after washing with 70% ethanol		
Han 2010	Third molar	DPSCs					Tooth was washed with PBS containing AA solution for 3 min after washing with 70% ethanol	Severed with pliers	
Haveleck 2013	Impacted third molar	DPSCs							
He 2014	Third molar	hDPSCs	18-22 y						
He 2013	Impacted third molar	hDPSCs	18-22 y						
He 2008	Impacted third molar	DPSCs	19-29 y						
Hilkens 2013	Third molar	DPSC-EZ DPSC-OG	15-20 y				Cleaned (not described)	Mechanically fractured with forceps	
Hirata 2010	Third molar upper						Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur	Barbed broach
Hoss 2013	Impacted third molar	DPSCs					Brushed by using sterilized dental burs		
Huang 2010 (a)	Third molar	DPSCs	14-22 y						
Huang 2010 (b)	Impacted third molar	DPSCs	16–24 y		Cell culture medium, serum-free				
Huang 2009	Left upper central incisor - traumatized Left upper lateral incisor - traumatized	hDPSCs	41 y					Endodontic file	
Huang 2008	Supernumerary tooth (a mesiodens)	DPSCs	20 y	Male	DPBS, on ice (Invitrogen Carlsbad, CA, USA)		Cleaned with DPBS (Invitrogen, Carlsbad, CA, USA)	Sterile hand-held high-speed drill. Cut around the curcumference of the teeth with chinsel.	Endodontic file
Ishkitiev 2012	Third molar	DPSCs					Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur	Sterile barbed broach
Ishkitiev 2010	Upper third molar	DPSCs					Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur	

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Jeon 2011	Impacted third molar	DPuSCs	16–18 y		D-PBS, on ice		Cleaning with DPBS containing 1% penicillin (Gibco), streptomycin (Gibco)		
Jin 2013	Third molar	hDPSCs	18-25 y						
Kadar 2009	Impacted third molar	DPSCs	18-26 y				Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur sterilized dental fissure bur	
Kanafi 2014		DPSCs	18-40 y						
Kanafi 2013 (a)		DPSCs	5-40 y						
Kanafi 2013 (b)		DPSCs	5 - 40 y						
Kanafi 2013 (c)		DPSCs	5 - 40 y				Washed 2–3 times with DPBS (Invitrogen, Calif., USA)		
Kanafi 2013(d)	Third molar	DPSCs	18-30 y						
Karaoz 2011	Third molar		17-25 y				Immerssion in pysiological solution containing antibiotics	Cut around the CEJ by using sterilized dental fissure bur	Sterile excavator
Karbanová 2011	Impacted third molar	DPSCs	17-23 y						
Karbanová 2010	Impacted third molar	DPSCs	17-23 y						
Kawanabe 2012	Premolar	DP	18-27 y					Cut using a dental turbine	
Kellner 2014	Impacted third molar	DPSCs	12-30 y						
Khanna-Jain 2012	Impacted third molar	DPSCs	Mean of 23 ± 2.5 years, 21–26 y						
Kim 2013	Third molar	hDPSCs							
Kim 2011	Premolar	DPSCs	18 and 19 y	Male			Cleaned (not described)	Cut around the CEJ using by sterilized dental disk	
Király 2011	Impacted third molar	DPSCs	19-35 y						
Király 2009	Impacted third molar	DPSCs	19–55 y						
Kolind 2014	Impacted third molar	DPSCs							
Koyama 2009	Impacted third molar		14-35 y				Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur	
Kraft 2010	Impacted third molar	PDSC-immature	21 y	Male					
		PDSC-mature	20 y	Female					

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Laino 2006	Third molar	DPSCs	19-37 y						Dentinal excavator or a gracey curette
Laino 2005		DPSCs	30-45 y				Pretreated a week before with professional dental hygiene. Before extraction, the dental crown was covered with 0.3% CHX gel (Forhans) for 2 min		Gracey curette
Lee 2014		DPSCs	18-39 y						
Lee 2012	Incisor	DPSCs	28 y 25 y	Male Female			Cleaned with DPBS		
Lee 2011 (a)	Third molar	hDPSCs							
Lee 2011 (b)	Impacted third molar	hDPSCs	18-22 y					Forceps	
Lee 2011 (c)	Molar	DPSCs			HBBS (Welgene, Dae-gu, Korea) supplemented with 3% AA (Gibco, Grand Island, NY) at 4°C			Dental high-speed unit	
Lee 2011 (d)	Third molar	DPSCs	18-35 y						
Lee 2010 (a)	Molar	hDPSCs							
Lee 2010 (b)	Third molar	DP-MSCs	17-38 y	Male Female			CHX solution	Hercules cutter	
Lee 2010 (c)	Premolar	DPSCs	18-30 y		Magnetically cryopreserved: Cryopreserved in a program freezer (ABI, Chiba, Japan) supplied with a slight magnetic field. Teeth were transported at4°C and then placed in a freezer at -5°C.	15 min	Cleaned with PBS		
					Then cooled at a rate of -0.5°C/min until -32°C. Then transferred to a freezer (MDF-11561; Sanyo, Osaka, Japan) and stored at -152°C.	84 h			
					Non-cryopreserved fresh teeth				
Lee 2008	Molar	hDPSCs					Cleaned (not described)	Cut around the CEJ by using sterilized dental fissure bur	

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Li 2011		hDPSCs	19-22 y	Male					
Lin 2011	Third molar	DPSCs	25 y 18 y	Male Female			Cleaned with PBS	Diamond burs	Forceps
Lindroos 2008	Impacted third molar	DPSCs	21-31 y						
Liu 2014		DPSCs							
Luo 2014 (a)		hDPSCs	18-25 y						
Luo 2014 (b)		hDPSCs							
Ma 2012	Third molar	DPSCs CDPSCs	18-28 y						
Makino 2013	Supernumerary	SNTSCs							
Mangano 2011	Third molar	DPSCs					Pretreatment for one week with professional dental hygiene. Before extraction dental crown covered with 0.3% CHX gel (Forhans, New York, NY) for 2 min		Dentinal excavator or a gracey curette
Mangano 2010		DPSCs					Pretreatment for one week with professional dental hygiene. Before extraction dental crown covered with 0.3% CHX gel (Forhans, New York, NY) for 2 min		Dentinal excavator or a gracey curette
Manikandhan 2010		DPSCs				6-48 h			Brooches
Marchionni 2009	Molar	DP-SCs	Mean of 35 y				Immerssion d in PBS containing AA: 100 U/mL penicillin, 100µg/mL streptomycin, 0.25 µg/mL amphotericin B. Then, immerssion in a 0,2% CHX solution.	Sterile diamond bur	
Martens 2012	Third molar	hDPSCs	18-24 y					Mechanically	
Martin 2013	Third molar	hDPSCs	Mean of 22.5 y		DMEM (Sigma-Aldrich, Steinheim, Germany) supplemented with 6% AA : 10,000 U/mL penicillin (Sigma Aldrich) 10 ng/mL streptomycin (Sigma-Aldrich) and 25 mg/mL amphotericin B (Sigma-Aldrich)	Immediately	Rinsed with PBS (Sigma-Aldrich). Periodontal tissues over the root surface were removed with a sterile surgical blade	Sterilized diamond bur	
Min 2011	Third molar	DPCs	20-25 y						

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Mokry 2010	Impacted third molar	DPSCs	18-27 y	Male Female					
Mori 2011	Third molar	DPSCs					Cleaned (not described)	Cut around CEJ by using sterilized dental fissure bur	
Murakami 2013	Third molar	DPSCs	18-29 y						
Murakami 2012	Third molar	DPSCs	18-29 y						
Muthna 2010	Impacted third molar	DPSCs	12-23 y	Male Female					
Nadeem 2013	Third molar	DPSCs							
Nakamura 2009		DPSCs							
Nam 2011	Third molar	hDPSCs	19-25 y						
Navabazam 2013	Third molar	DPSCs	15-32 y						
Nawi 2013		DPSCs			HBSS	Immediately	Periodontal and gingival tissues were scrapped off from the tooth surface using sterile surgical blade. Surface was cleaned with iodone and 70% ethanol. Then, washed with DPBS.	Cut a the CEJ by using hard tissue cutter (Exact, Düsseldorf, Germany)	Barbed roach (Dentslpy, Germany)
Nesti 2011	Molar	DPSCs	18-35 y						Gracey curette
Neuss 2008	Impacted third molar	DPSCs					Cleaned (not described)	Cut around the CEJ by using sterilized dental bur	
Niu 2014	Third molar	hDPSCs	18-25 y						
Oancea 2013		DPSCs	12-17 y				Pretreatment for a week with professional dental hygiene		Dentinal excavator or a gracey curette
Okamoto 2009	Third molar	DPSCs	22-26 y						
Osathanon 2014	Impacted third molar	DPSCs							
Osathanon 2011	Impacted third molar	DPSCs							
Paino 2010	Molar	DPSCs							
Palumbo 2013	Third molar	hDPSCs							
Pang 2013	Third molar	DPSCs	16-22 y						Barbed broach

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Papaccio 2006		SBP-DPSCs	21-45 y				Pretreatment for a week with professional dental hygiene. Before extraction, the dental crown was covered with 0.3% CHX gel (Forhans, NY) for 2 min.		Dentinal excavator or a gracey curette
Park 2013	Impactec third molar	hDPSCs	18-35 y						
Patil 2014	Third molar	DPSCs	16-18 y	Male			Rinsed several times with DPBS containing 1% penicillin and 1% streptomycin	Bone forceps	
Pereira 2012 (a)	Molar	N-hDPSCs I-hDPSCs	17-43 y					Sterilized diamond discs and dental surgical elevator	Sterile dentinal excavator
Pereira 2012 (b)		DPSCs-N DPSCs-I	17-43 y					Sterilized diamond discs (KG Sorensen, ref. 7020, Barueri, São	Sterile dentinal excavator
Perry 2008	Third molar	DPSCs	18-30 y		20mL of one of three collection/transport solutions: HTS (BioLife Solutions, Bothell, WA) MesenCult basal medium (Stem Cell Technologies, Vancouver, Canada). PBS (Sigma Chemical, St. Louis, MO)	Immediately	Washed with sterile PBS, followed by immersion in 1% PVP-I for 2 min, immersion in 0.1% sodium thiosulfate in PBS for 1 min, and another wash in sterile PBS		
Picchi 2013	Molar								Gracey curette
Pierdomenic 2005	Molar	DP-MSCs	Mean of 40 y	Male Female		Immediately	Immerssion in physiological solution containing antibiotics	Bone forceps	
Ponnaiyan 2012	Impacted third molar	DPSCs	18-22 y						
Riccio 2010	Third molar	DPSCs	18-35 y						
Rizk 2013 (a)	Premolar	DPSCs						Bone cutter	
Risk 2013 (b)	Premolar	hDPSCs							
Rodriguez-Lozano 2013	Impacted third molar	DPSCs			DMEM supplemented with 10 % of FCS, 100 U/mL penicillin and 100 µg/mL streptomycin	24 h			
Rodríguez-Lozano 2012	Impacted third molar	DPSCs	Mean of 29 y, 21-45 y	Male Female					

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Ryu 2009	Molar	hDPSCs							
Sakai 2012	Third molar	DPSCs	18-30 y						
Schiraldi 2012	Third molar	DPSCs	21-45 y						
Seifrtova 2013	Impacted third molar	DPSCs							
Seifrtova 2012	Impacted third molar	DPSCs							
Seo 2013	Impacted third molar	DPSCs	20-28 y				Cleaned (not described)	Cut around CEJ by using sterilized diamond stones	
Shafiei 2014	Third molar	DPSCs	20-25 y					Cut around CEJ by using fissure bur	
Shekar 2012	Impacted third molar Premolar	DPSCs			α-MEM supplemented with antibiotics: 100 IU penicillin,	Immadiately	Washing with DPBS	Chisel and mallet	Forceps and a spoon excavator (2 mm diameter)
Shi 2002	Impacted third molar	DPSCs							
Sollazzo 2011	Third molar	DPSCs	20-25 y						Gracey curette
Son 2006	Molar	hDPSCs					Cleaned (not described)	Cut around JEC by using sterilized dental fissure bur	
Spath 2010	Third molar	DPSCs	22-35 y				Treatment one week before extraction with professional dental hygiene. Before extraction, dental crowns were covered with a 0.3% CHX gel (Forhans, NY, USA) for 2 min		Dentinal excavator or a gracey curette
Stevens 2008	Premolar	hDPSCs							
Stokowski 2007	Impacted third molar	DPSCs	18-40 y				Cleaned (not described)	Vise	
Struys 2013	Third molar	DPSCs	16-19 y					Fractured mechanically	Forceps
Struys 2011	Third molar	DPSCs						Fractured mechanically	Forceps
Suchanek 2013	Semi-erupted third molar	DPSCs	23 y	Male	HBSS (Gibco, UK)	Immediately		Luer's forceps	Extirpation needle or tweezers
	Third molar		22 y	Female					

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Suchanek 2009	Third molar	DPSCs	12-23 y	Male Female	HBSS (Gibco, Scotland)	Immediately		Luer's Forceps	Extirpation needle or sharp excavator (Henry Schein, UK)
Suchanek 2007	Impacted third molar Third molar Premolar	DPSCs	15-23 y		HBSS (Gibco, Scotland)	Immediately		Skive or luer's Forceps	Excavator (Henry Schein Inc., UK) and sharp needle
Suh 2014	Third molar	DPSCs	19-40 y					Cut around CEJ by using sterilized dental fissure bur	
Suri 2008	Premolar Molar	HDPCs						Chisel and mallet	Sterile spoon excavator and tweezers
Suzyki 2011	Third molar	DSCs	14 y 28 y	Male Female					
Tamaki 2013	Third molar	DPSCs	16-28 y					Cut around CEJ by using sterilized dental fissure bur	
Tammaro 2014	Impacted third molar	hDPSCs	18-22 y					Forceps	
Tandon 2010	Premolar	DPSCs				30 min	Rinse with 0.2% CHX for 60 sec		Barbed broach
Tirino 2012	Third molar	DPSCs					Treatment with professional dental hygiene. The dental crown is covered with 0.3% CHX gel for 2 min (Forhans)		Dentinal excavator or a gracey curette

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Tomic 2011	Third molar	DP-MSCs							
Tom-Kun 2011		hDPSCs			Serum-free culture medium	Immediately			
Trubiani 2012	Premolar						Pretreatment for a week with professional dental hygiene. Rinsed four times in PBS containing penicillin and streptomycin.	Cylindrical diamond bur (Intensiv, Grancia, Switzerland) mounted on a high-speed handpiece (Bora L; Bien-Air, Bienne, Switzerland) with water-spray cooling	Sterile dentinal excavator
Trubiani 2010	Premolar	DP-MSCs					Pretreatment for a week with professional dental hygiene. Rinsed four times in PBS containing penicillin and streptomycin.		Sterile dentinal excavator
Trubiani 2007			24-30 y				Rinsed four times in PBS containing penicillin and streptomycin		
Uchiyama 2009	Third molar	DPSCs	37 and 42 y	Female				Hammer	
Um 2011	Third molar	DPSCs	20-24 y	Male					
Vandomme 2014	Third molar Premolar	DPSCs			Realized (not described)		Cleaned (not described)		

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Varga 2011	Impacted third molar	hDPSCs	Mean of 27 y	Male Female	Sterile physiologic saline with gentamicin (Lék, Slovenia)	Immadiately	Rinsed with PBS (Oxoid, GB)	Cut around CEJ by using Luer's forceps	Excavator
Vasandan 2014	Impacted third molar	DPSCs	17-28 y				Washed with DPBS (Gibco, Grand Island, NY, USA) containing AA		
Ventura 2007	Molar	DPhMSCs					Immerssion in physiological solution containing antibiotics	Bone forceps	
Vishwanath 2013		DPSCs	Less than 25 y		FBS	Immediately	Cleaned (not described)	Sterilized dental bur	Small size broach and a blunt non cutting forceps
Wada 2009	Premolar	DPSCs							
Wang 2014	Impacted third molar	hDPSCs	19-28 y						
Wang 2013 (a)		DPSCs	14-25 y						
Wang 2013 (b)	Premolar	DPSCs	12-13 y						

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Wang 2012	First premolar	DPSCs	18-20 y						
Wang 2010	Third molar	DPSCs	15-25 y						
Weszl 2012	Impacted third molar	DPSCs	18-26 y					Cut around CEJ by using sterile dental fissure bur	
Woods 2009	Third molar	DPSCs	15-30 y		PBS (Sigma Chemical, St. Louis, MO)	24 h	Washed with sterile saline, exposure to 1% PVP-I for 2 min, 0,1% sodium thiosulfate in PBS for 1 min and		Curette
Yan 2010		DPSCs							
Yu 2009	Premolar	DPSCs					Cleaned with dental soaler to remove attached soft tissue	Splitting the teeth at the CEJ sterile dental drill	
Zhai 2013	First premolar	hDPSCs							

Author and year	Tooth type	Cell type	Age	Gender	Tooth storage methods	Tooth storage time	Dental surface cleaning	Dental section methods	Pulp removal methods
Zhang 2011	Third molar	DPSCs	16 y	Female				Vise	
Zhang 2010	Third molar	DMCs	16 y	Male					
Zhang 2008 (a)	Impacted third molar	DPSCs	22 y	Male					
Zhang 2008 (b)	Impacted third molar	DPSCs	22 y	Male					
Zhang 2006	Impacted third molar	DPSCs	18-24 y		α-MEM (Gibco BRL, Life Technologies B.V. Breda, The Netherlands) 0.5 mg/mL of gentamicin (Gibco BRL), 3mg/mL amphotericin B (Gibco BRL)		Cleaned (not described)	Cut around the CEJ by using a high-speed dental drill	
Zhao 2011	Third molar	hDPSCs	18-35 y	Male Female					
Zhao 2006	Lower premolar	hDPSCs							
Zhou 2014	Impactec third molar	DPCs	18-30 y						

6 **Artigo 3**

Tolerance of dental pulp stem cells from permanent and deciduous teeth against oxidative stress: A pilot study

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Abstract:

A number of diseases such as acute myocardial infarction, cerebral ischemia and diabetes are closely related to environments with high levels of oxidative stress, due to imbalances between the production of ROS and the ability to neutralize these species by the antioxidant system. Currently, several studies have been conducted in order to develop therapeutic improvements in this context. Thus, the aim of this study was to investigate the tolerance of dental pulp stem cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHEDs) when subjected to an oxidative stress environment. The cell viability analysis of DPSCs, SHEDs and pulp fibroblast (PF) was conducted after the insult with H_2O_2 for 2, 6 and 12 hours, through the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The results suggest that the cell viability of DPSCs and SHEDs submitted to H_2O_2 is dose and time dependent. SHEDs P3, seems to have the capacity to adept to a oxidative stress environment, showing increase in cell viability when summited to 50 µM of H_2O_2 This is follow by the increase of SOD activity. SHEDs P10 and DPSCs P3 and P10 showed lower capacity to increase cell viability when submitted an oxidative stress environment. To the best of our knowledge this is the first time that DPSCs and SHEDs are evaluated for their tolerance to an oxidative stress environment. However it is important to highlight the limitations of this study, since it is a pilot study and more analysis will be conducted in order to investigate SOD activity in DPSCs P3 and P10 and SHEDs P3, as well as other antioxidant enzymes.

Key words: Dental pulp stem cells; Stem cells from human exfoliated deciduous teeth; Reactive oxygen species; Oxidative stress.

1. Introduction

Reactive oxygen species (ROS) and nitrogen are produced naturally during cell function (Valko, Leibfritz et al. 2007). Although the majority of ROS seems to be generated by endogenous production, exogenous sources such as ultraviolet radiation, ozone, pollutants (Balaban, Nemoto et al. 2005), heavy metals (Jaishankar, Tseten et al. 2014), smoking and alcohol ingestion (Gorlach, Dimova et al. 2015), may also be responsible by triggering the formation of free radicals (Balaban, Nemoto et al. 2005).

ROS can perform a variety of important roles, acting as second messengers, participating in cellular signaling pathways (Haddad 2002, Haddad 2002), and directly influencing gene transcription factors (Haddad 2002). However, depending on ROS relative concentration they are able to generate tissue damage (Uccelli, Moretta et al. 2008, Monaghan, Metcalfe et al. 2009), and have been related to various types of diseases such as osteoporosis (Sun, Yang et al. 2013) and diabetes (Crujeiras, Diaz-Lagares et al. 2013). Thus the balance maintenance between production of free radicals and the antioxidant defenses is a prerequisite for the organism normal behavior, avoiding therefore pathogenesis processes (Valko, Leibfritz et al. 2007). The imbalance between free radicals concentration and the antioxidant system removal capacity is defined as oxidative stress (Halliwell and Whiteman 2004, Monaghan, Metcalfe et al. 2009).

In order to prevent the formation of an environment with oxidative stress, an antioxidant system, composed by two pathways, enzymatic and non-enzymatic, are present and holds the capacity to neutralize ROS (Valko, Leibfritz et al. 2007). This activity prevents possible oxidative damage amplification that could culminate in irreparable systemic damage (Valle-Prieto and Conget 2010). The non-enzymatic system is constituted by endogenous origin and diet components (Valko, Leibfritz et al. 2007, Zhou, Xu et al. 2013). In turn, the enzyme system is widespread throughout the organism, in the intra and extracellular medium (Valko, Leibfritz et al. 2007), and includes enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Valko, Leibfritz et al. 2007, Zhou, Xu et al. 2013).

Thus, due to the formation of an oxidative stress environment and its the possible negative consequences (Halliwell and Whiteman 2004, Monaghan, Metcalfe et al. 2009) it is necessary to develop new strategies to overcame this issue. In this context, mesenchymal stem cells have been studied in order to develop new

approaches in pathologies associated with oxidative stress, such as acute myocardial infarction, cerebral ischemia and diabetes (Valle-Prieto and Conget 2010). The literature has indicated that stem cells could present a higher antioxidant activity and better adaptation to an oxidative stress environment when compared to other cell types (Caplan 2009, Valle-Prieto and Conget 2010).

In this sense stem cells have shown higher levels of total glutathione (GSx) and methionine sulfoxide reductase A, this last one being a crucial enzyme for the repair of oxidized protein and for the recovery of methionine residues. In addition it has been shown that mesenchymal stem cells express enzymes required for DNA repair. This stem cell, therefore, could present the basic enzymatic and non-enzyme machinery to detoxify reactive species and to correct proteome and genome damage caused by oxidative stress, ensuring the efficient management of oxidative stress (Valle-Prieto and Conget 2010). However, studies about the potential of stem cells to minimize oxidative stress are still very scarce.

Human dental pulp stem cells from permanent (DPSCs) and exfoliated deciduous (SHEDs) teeth are examples of mesenchymal stem cells. These cells can be easily obtained due to orthodontic, periodontal and dental caries reasons, as well as from natural exfoliation of deciduous (Chen, Sun et al. 2012), being therefore isolated by noninvasive methods and able to promote fast proliferation *in vitro* (de Mendonca Costa, Bueno et al. 2008). Despite the accessibility of DPSC and SHEDs, to the best of our knowledge, the literature has not investigated specifically the behavior of these cells when subjected to oxidative stress. And if these cells could be applied for oxidative stress environmental management in order be applied in cell therapy. Thus, the aim of this study was to investigate the cell viability and SOD rates of DPSCs and SHEDs when subjected to an oxidative stress environment.

2. Methodology

2.1 Isolation of DPSCs, SHEDs and PF

This study was approved by the Ethics Committee of the Faculty of Dentistry, Federal University of Pelotas, under the protocol number 38/2013. DPSCs, SHEDs and pulp fibroblasts (PF) isolation were performed as recommended by Kerkis et al. (Kerkis, Kerkis et al. 2006). Teeth were obtained from healthy patients that had dental extraction indication. Deciduous teeth were obtained from patients with an average of 8.6 years, with the minimum of 7 and maximum of 10 years old. For the isolation of

DPSCs permanent teeth were obtained from patients with an average of 23.18 years, minimum age of 16 and maximum 32 years. PF were obtained from permanent teeth, with an average of 24.7 years, being 24 year the minimum age and 25 the maximum. In all cell types, the great percentages of donors were females.

The surface of freshly extracted teeth was cleaned with 0.2% chlorhexidine solution. Subsequently, two grooves were held in the tooth surface, one in the occlusal and another one in the proximal face in the long axis of the tooth, using a diamond bur trunk-conical (number 4138 - KG Sorensen), in high-speed handpiece under constant irrigation with Milique sterile water. Teeth were than stored in falcons with cell culture medium [Dulbecco's Modified Eagle Medium (DMEM) (Cultilab, Campinas, SP, BR)], and kept in ice for a maximum period of 2 hours prior to cell isolation. Once in the laboratory in the teeth proximal groove, a chisel was adapted and with a hammer a rapid impact was provided so that the impact force generated could create a fracture line in the tooth structure revealing the pulp tissue.

When the dental pulp chamber was accessed, the pulp tissue was removed with dentin curettes and placed in a petry plate with 200 µL of medium culture. With a scalpel blade, number 15, the pulp tissue was fragmented into explants of about 2 mm^3 and transferred to the surface of a 6-well plate, fixed on grooves previously made with a scalpel blade in the bottom of the well plate in order to increase adhesion. In each well 2 mL of Dulbecco's Modified Eagle's Medium-Ham's/F12 (DMEM-Ham's/F12) (Gibco/Invitrogen, Carlsbad, Calif., USA) supplemented with 15% fetal bovine serum (FBS) (HyClone, Logan, Utah, USA), 1% antibiotics (Gibco/Invitrogen, Carlsbad, Calif., USA) and 1% essential amino acids (Gibco/Invitrogen, Carlsbad, Calif., USA) for DPSCs and SHEDs, and Dulbecco's Modified Eagle Medium (DMEM) (Cultilab, Campinas, SP, BR) supplemented with 10% of FBS (Gibco/Invitrogen, Carlsbad, Calif., USA) for PF, was added to allow cell migration. Then plates were incubated at 37°C in an atmosphere of 95% humidified air and 5% CO_2. Cultured explants were analyzed weekly in an inverted microscopic (TS100 TS100-F da Nikon) in order to monitor cell migration and density.

2.2 Dose response assay

The dose response assessment of DPSCs, SHEDs, FP and mouse fibroblast cell line (3T3/NIH) (obtained from the Rio de Janeiro Cell Bank - PABCAM, Federal University of Rio de Janeiro, RJ, Brazil) was determined by creating an oxidative stress

environment with hydrogen peroxide (H_2O_2). Cell viability was analyzed by the colorimetric MTT assay (3- [4,5-dimethyl-thiazol-2-yl] -2,5-diphenyltetrazolium bromide), where this soluble compound is reduced to insoluble formazan crystals (Nedel, Campos et al. 2012). DPSCs and SHEDs in passages 3 (P3) and 10 (P10), PF and NIH/3T3, were seeded at a density of $2x10^4$ cells/well and 100 µl/well volume in a 96 well plates. Subsequently, after 24 h of incubation at 37°C in an atmosphere of 95% humidified air and 5% CO_2 100 µL of culture medium and H_2O_2 at a final concentrations of 50, 100, 250, 500 µM was added. After 2, 6 and 12 hours of incubation 20 µL of MTT (5 mg MTT/ml of culture medium) was added to the culture medium of each well and incubated for 3 hours. 200 µL of dimethyl sulfoxide (DMSO) was added to each well, and the formazan was solubilised on a shaker for 5 min at 100 × g. The absorbance of each well was read on a microplate reader (MR-96A, Mindray Shenzhen, China) at a wavelength of 450 nm. All observations were validated by at least two independent experiments and each experiment was performed in quadruplicate.

2.3 Oxidative Stress Assay

Briefly, cells were seeded at a density of 5.81×10^5 cell per well in a volume of 500 µL in 24-well plate and grown at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air for 24 h before the oxidative stress assay. The culture medium was removed and cells were incubated with 500 µL of culture medium and H_2O_2 in a final concentration of 100, 250, 500 µM for 24 hours. After incubation the medium was removed, and 300 µL of phosphate-buffered saline (PBS) (Gibco/Invitrogen, Carlsbad, Calif., USA) was added, the cell monolayer was then gently detached and stored at - 20 ° C. Before the enzymatic activity assessment samples were submitted 3 times to thawing and immediate freezing, in order to promote the disruption of the cell membrane.

2.3.1 Protein Assay

The full protein concentrations were determined using the method described by Lowry et al. (Lowry, Rosebrough et al. 1951) with bovine serum albumin as the standard.

2.3.2 Superoxide Dismutase Activity

Superoxide dismutase activity was determined using the RANSOD kit (Randox Labs., USA), which is based on a procedure described by Delmas-Beauvieux et al (Delmas-Beauvieux, Peuchant et al. 1995). This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with 2-(4-iodophenyl)- 3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a formazan dye, which can be assayed spectrophotometrically at 492 nm, at 37 °C. The inhibition of the production of the chromogen is proportional to the activity of SOD present in the sample; one unit of SOD causes 50 % inhibition of the rate of reduction of INT under the conditions of the assay.

2.4 Data analysis

Cell viability was analyzed by analysis of variance (ANOVA) flowed by Tukey post-hoc test. The value of $P<0.05$ was considered significant. Graphic for cell viability and SOD was developed using GraphPad Prism Program 4.00 version (GraphPad Software, San Diego, USA).

3. Results and discussion

Science has invested consistently in the development of therapeutic strategies for oxidative stress-related diseases, such as acute myocardial infarction (Chen, Fang et al. 2004), diabetes (Lee, Seo et al. 2006, Ezquer, Ezquer et al. 2008) and Alzheimer (Perry, Cash et al. 2002). In the interest of improving cell therapy for these diseases, Valle Prieto et. al (2010) reported that bone marrow mesenchymal stem cells are highly resistant to death induced by oxidative stress, being able to scavenge ROS and reactive nitrogen species (RNS), and therefore efficiently manage oxidative stress *in vitro* (Valle-Prieto and Conget 2010).

In this context, we investigated the behavior of DPSCs and SHEDs comparing them to dental pulp fibroblast (PF), non stem cells when exposed to an oxidative stress environment simulated by H_2O_2 at different concentrations (50, 100, 250 and 500 µM) for 2, 6 and 12 hours. The focus on these specific stem cell types is due to the fact that the teeth structure are not considered an essential organ (Sharpe and Young 2005). Furthermore teeth can be easily accessed due to frequent therapeutic indication for teeth extraction, as a consequence of periodontal and caries diseases,

as well as extractions during orthodontic treatment and the natural exfoliation of deciduous teeth (Chen, Sun et al. 2012).

In the present study, DPSCs P3 cells showed no statistical differences between control and the different concentrations (50, 100, 250 and 500 µM) of H_2O_2 tested in 2 hours (P=0.186). However, as the time of exposure increased to 6 hours, the group treated with 50 µM of H_2O_2 showed the higher cell viability between all groups. The increase of H_2O_2 to 100 and 250 µM decreased slightly cell viability, however it was maintained statistically higher than the control group. The only group that showed cell viability lower then the control group was the one treated with 500 µM of H_2O_2 (P=0.000). As the time of exposure continued to rise to 12 hours, DPSCs P3 cell treated with 50 and 100 µM of H_2O_2 showed cell viability similar to control, with cell viability decreasing with 250 and 500 µM (P= 0.001)(Figure 1).

SHEDs in the same cell passage (P3) shows different behavior toward oxidative stress stimulus. In all periods of time tested (2, 6 and 12 hours) cell viably was always higher in the group insulted with 50 µM of H_2O_2 (P=0.003; P=0.016; P=0.002, respectively). Cell exposed to 100 µM of H_2O_2, in all times, showed no statistical differences with the control group, and the same was observed with the concentration of 250 µM in 6 hours of oxidative stress insult. 250 µM (in 2 and 12 hours), and 500 µM (in 2, 6 and 12 hours), showed decreased viability when compared to controls (Figure 3). The results described for DPSCs and SHEDs in P3 shows different behavior of stem cells towards oxidative stress. SHEDs seems to be able to maintain its higher cell viability with 50 µM within 12 hour of exposure to H_2O_2 will DPSCs with 12 hours of exposure loses its viability capacity in the same insult concentration. These findings could be related to the process that permanent and exfoliated teeth are undergoing when DPSCs and SHEDs are obtained. When SHEDs are isolated, teeth are in their final process of exfoliation, and therefore cell have been continually exposed to oxidative stress and pro-inflammatory cytokines in the process of root resorption. This could partially explain way SHEDs maintain high viability in long period of exposure to oxidative stress on the contrary to DPSCs from permanent teeth, which in normal condition are not exposed to reabsorption environment.

DPSCs in P10 showed no statistical differences with 2 hours of exposure to different concentrations of H_2O_2 (P=0.502). Within 6 hours of exposure to 50, 100 and 250 µM of H_2O_2 cell viability was statistically similar to the control group. Lower cell viability

was observed only when cells were exposed to 500 µM of H_2O_2 (P=0.000). When time of exposure was increased to 12 hours, cells stimulated with 100 µM started to show increase in cell viability when compared to the control. With 50 and 250 µM of H_2O_2 cell viability was shown similar to the control group, and cell viability decreased with 500 µM of H_2O_2 (P=0.033) (Figure 2). SHEDs P10 showed no statistical differences between groups when exposed to 2 and 6 hours of H_2O_2 (P=0.232; P=0.065, respectively). When the exposure increased to 12 hours the concentrations of 50, 100 and 250 µM of H_2O_2 showed equal viability with the control group and viability decreased with 500 µM (P=0.000) (Figure 4).

When pulp fibroblasts, cells in there final differentiation process without, therefore, *stemness* proprieties, were treated with H_2O_2, lower cell viability was observed with 500 µM in 2 and 6 hours of exposure. All groups, in there different period of exposure (2, 6 and 12) presented no statistical differences with the control group (P=0.000; P=0.000; P=0.575, respectively) (Figure 6).

SOD activity in SHEDs P3 showed a peak tendency of activity when cells were treated with 50 and 100 µM of H_2O_2, showing a tendency to promote greater enzymatic activity compared to the control group (Figure 5). When evaluating PF exposed to different concentration of H_2O_2, cells showed no tendency to increase SOD activity and decreased its activity with 500 µM.

It can be speculated that cell viability tends to present more sensitivity to high concentrations of H_2O_2 then in the increase of exposure time. This could be an important aspect since in injured tissue an environment of oxidative stress can persist for long periods of time, and therefore, cells with and without *stemness* properties seems to develop this knowledge in order to survive.

Currently two main pathways for ROS function have been related to mesenchymal stem cell. One pathway concerns the damaging effect of ROS, which are believed to be the main factor for stem cell aging (De Barros, Dehez et al. 2013). This observation is based on the consumption that stem cells can be expanded efficiently under hypoxic conditions *in vitro* wile retaining multipotency and when H_2O_2 is introduced in the medium it induces premature senescence of these cells. On the other hand intracellular ROS have been shown to participate in the regulation of stem cell fate (Naka, Muraguchi et al. 2008), acting as secondary messengers, participating in signaling pathways (Haddad 2002, Haddad 2002) and directly influence transcription factors genes (Haddad 2002). Low concentrations of

exogenous H_2O_2 induces moderate levels of ROS in stem cells, which have been associated with quiescent mesenchymal stem cells. The increase of ROS levels can induce stem cell proliferation (Lyublinskaya, Borisov et al. 2015) and migration through the activation of extracellular-signal-regulated kinase (ERK) 1/2 and Jun-1/2 pathways (Sart, Song et al. 2015).

According to the possible roles of ROS in cell proliferation, our results indicate that DPSCs in P3 seems to be stimulated to increase cell proliferation in 6 hours were this effect is lost when oxidative stress is stimulated for 12 hours. On the contrary DPSCs P10 seem to need a burst of oxidative stress stimulation when compared to P3, were the increase of proliferation was observed only with 12 hours with a H_2O_2 concentration of 100 µM. In the same sense SHED P3 was stimulated to proliferate by H_2O_2 in a concentration of 50 µM through 2, 6 and 12 hours of stimulation. This indicates the potential of this cell for cell therapy application. On the contrary SHEDs P10 did not show the capacity to be stimuli to proliferate in the different concentration of H_2O_2 tested. The same was observed for fibroblast cell. The literature has shown that as stem cells proliferate and advance into higher cell passages, cells begin naturally to differentiate, since these cells are removed from there niches they are devoid of the inducing signal for multipotency maintenance (Langer and Vacanti 1993), and therefore we questioned if cells in different cell passages could present different oxidative stress tolerance. Therefore, the results described above indicates that SHEDs P3 have more potential of stemness and therefore can be stimulated with lower concentrations and for lower periods of exposure time. Pulp fibroblasts are somatic cells completely differentiated when compared to DPSCs and SHEDs and therefore showed no capacity to proliferate with the increase of oxidative stress in the environment.

All cell types showed decrease in cell viability when exposed to 500 µM in all period of time tested. As mentioned above excessive concentration of ROS can lead to oxidative stress in stem cells, disrupting its adhesion through the down-regulation of the activated focal adhesion kinase (FAK), Scr, and integrin’s expression (Ren, Wang et al. 2015). Also it can lead to DNA damaging, reduction of telomere length leading therefore to cell senescence (Borodkina, Shatrova et al. 2014).

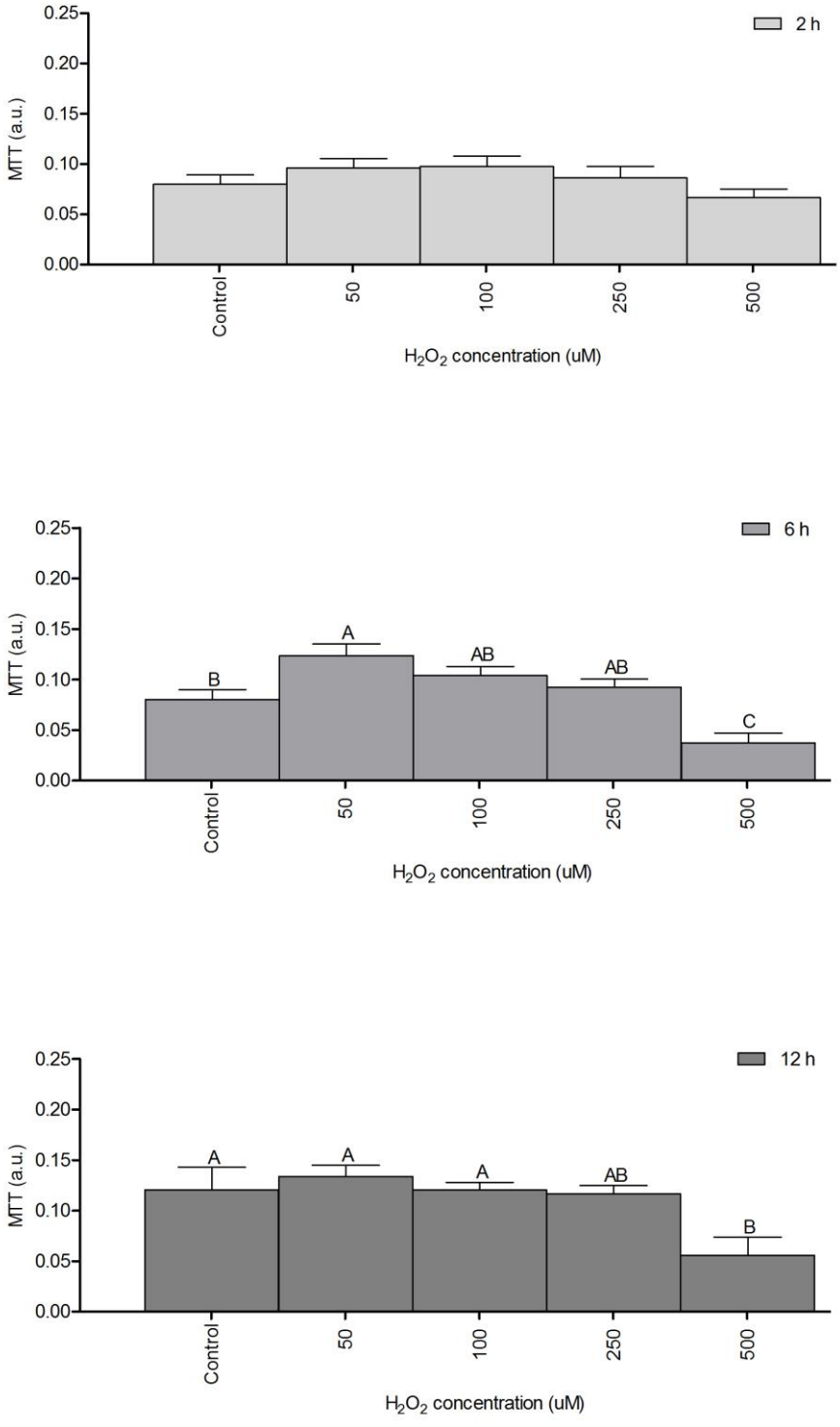


FIGURE 1: DPSCs P3 viability when exposed to different concentrations of H_2O_2 (50, 100, 250, and 500 µM), for a time period of 2, 6 and 12 hours.

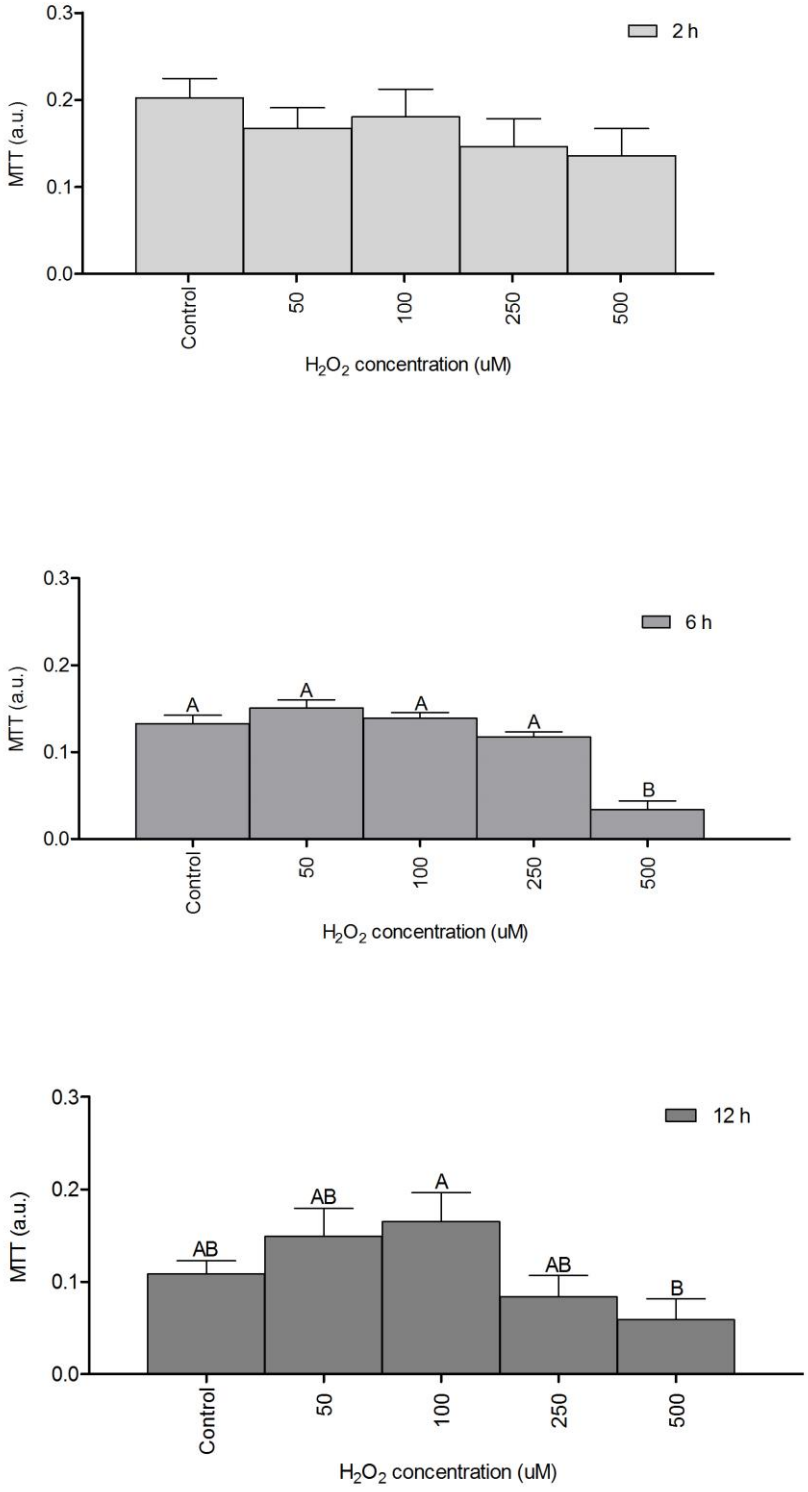
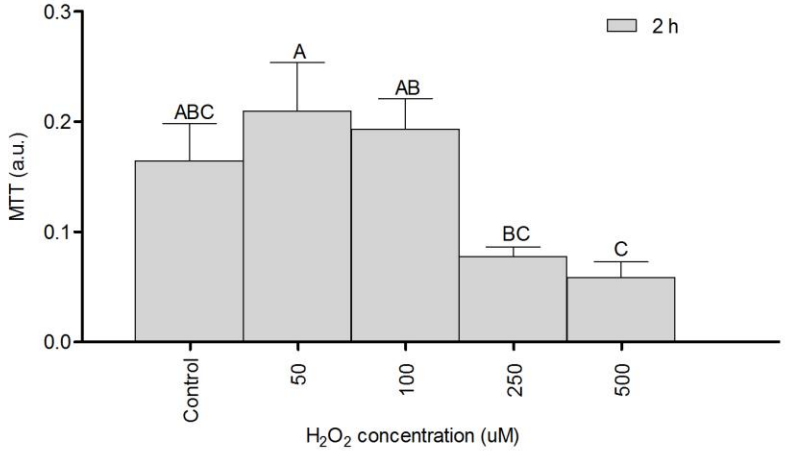
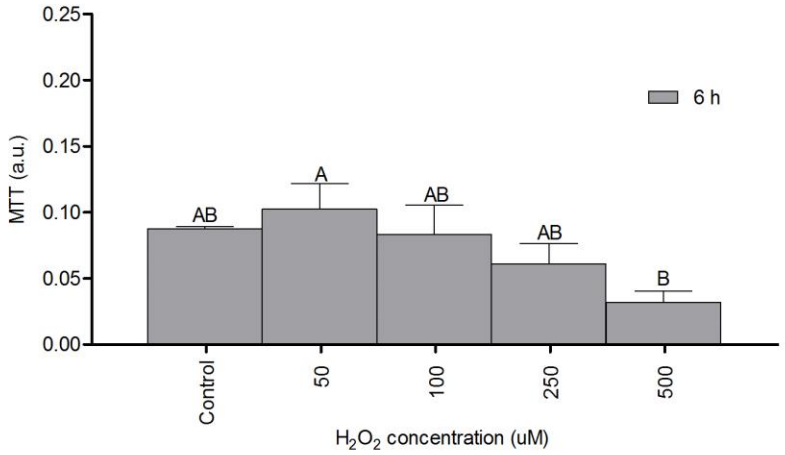


FIGURE 2: DPSCs P10 viability when exposed to different concentrations of H_2O_2 (50, 100, 250, and 500 µM), for a time period time of 2, 6 and 12 hours.





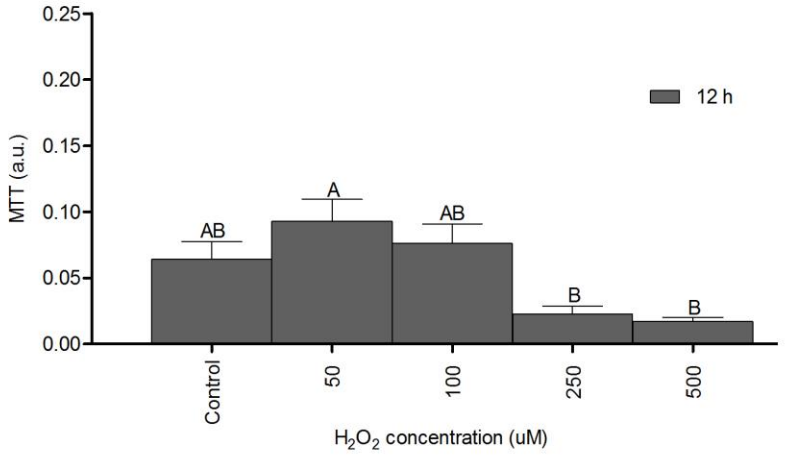


FIGURE 3: SHEDs P3 viability when exposed to different concentrations of H_2O_2 (50, 100, 250, and 500 µM), for a time period of 2, 6 and 12 hours.

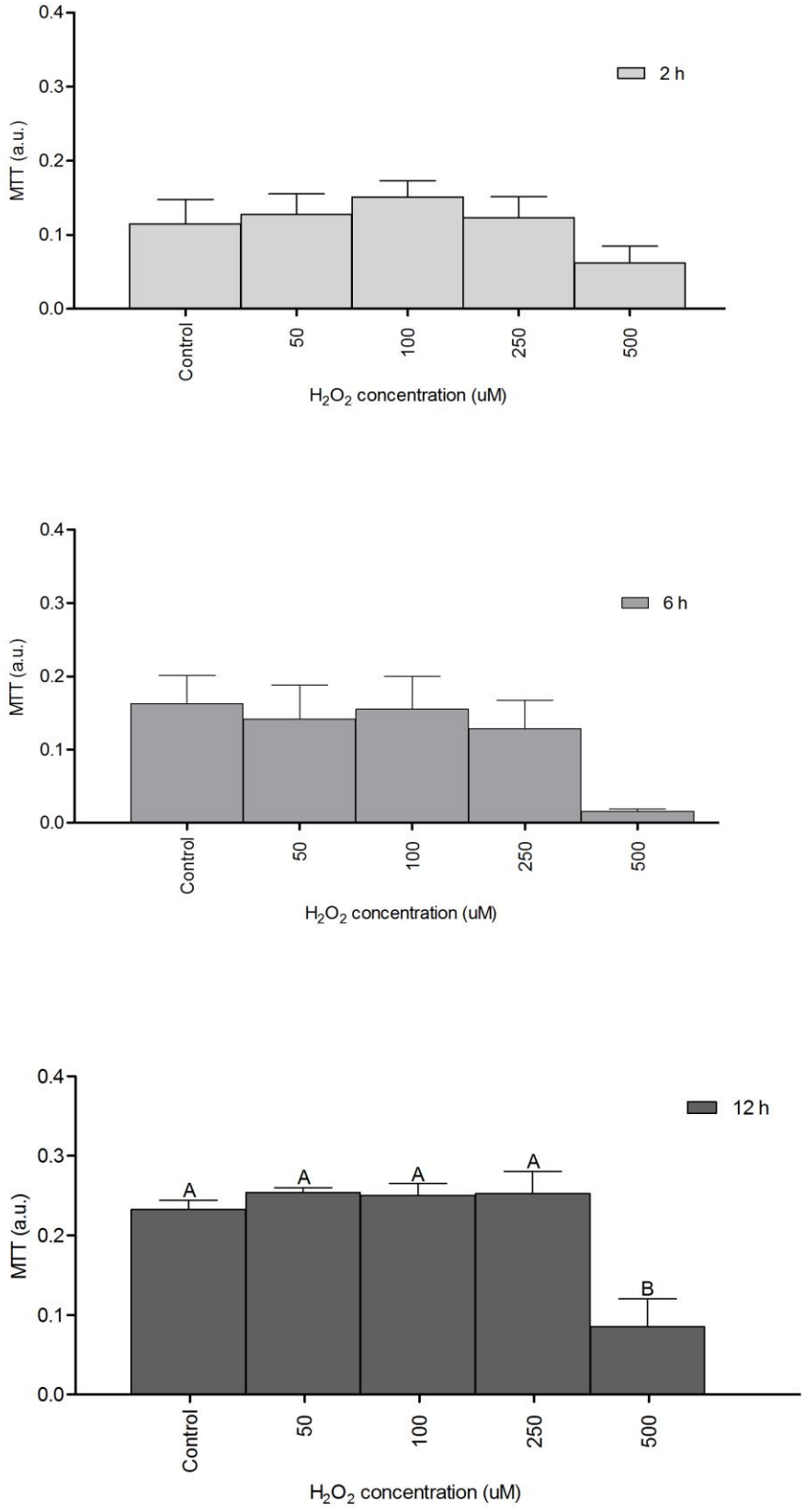


FIGURE 4: SHEDs P10 viability when exposed to different concentrations of H_2O_2 (50, 100, 250, and 500 µM), for a time period of 2, 6 and 12 hours.

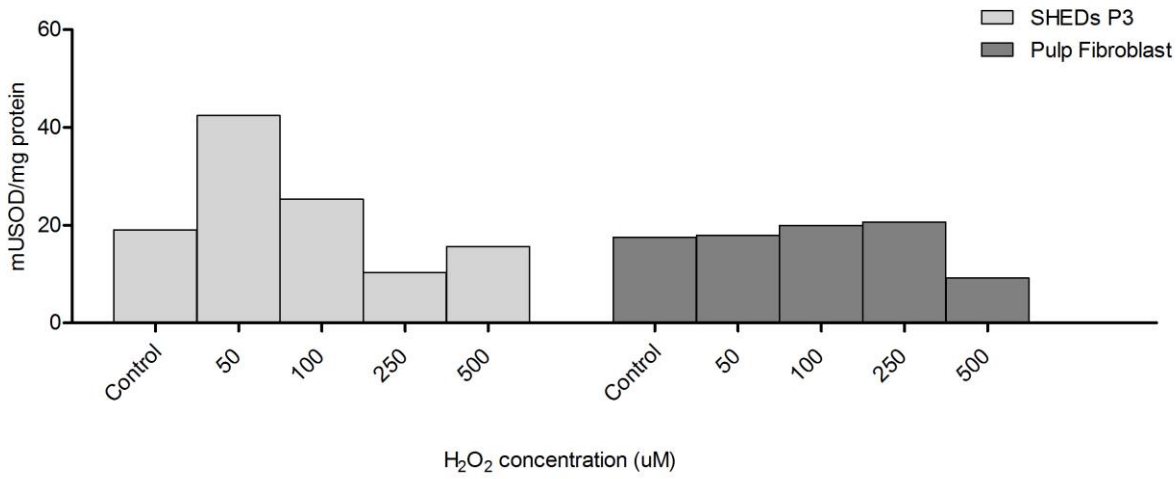


FIGURE 5: SOD enzyme activity in SHEDs P3 and Pulp Fibroblast, when exposed to different concentrations of H_2O_2 (50, 100, 250 and 500 µM), for 12 hours.

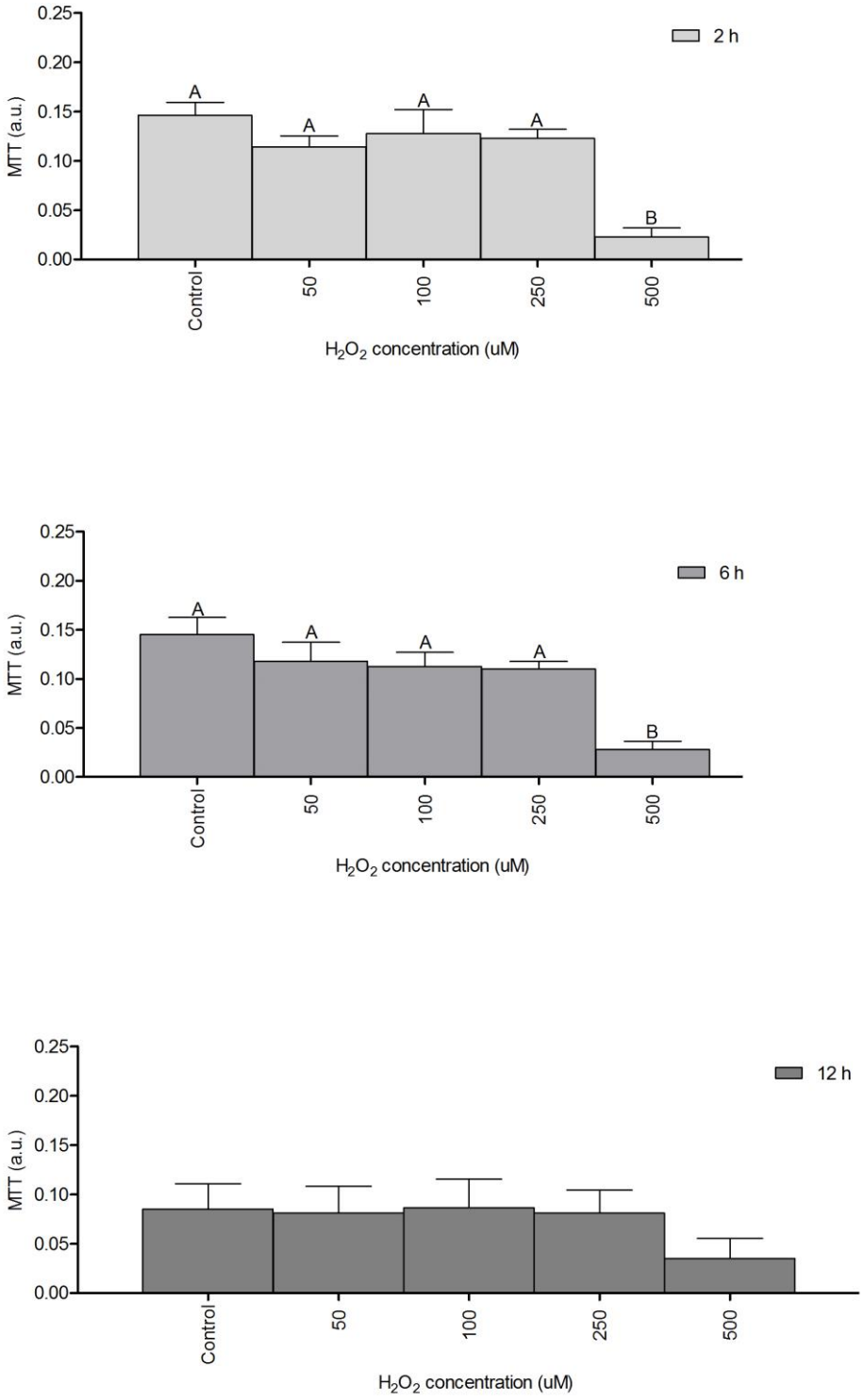


FIGURE 6: PF viability when exposed to different concentrations of H_2O_2 (50, 100, 250, and 500 µM), for a time period of 2, 6 and 12 hours.

To the best of our knowledge this is the first time that DPSCs and SHEDs are evaluated for their tolerance to an oxidative stress environment. However since this

is a pilot study, it is worth mentioning that further testing towards oxidative stress management potential of DPSCs and SHEDs will be conducted. SOD activity for DPSCs P3 and P10 and SHEDs P10 will be conducted. Also, CAT and GPx activities and TBARS assay for all cell types will be achieved. However, based on the analysis of the above data, it is expected that higher values for antioxidant enzymatic activity well be observed in the groups exposed to H_2O_2 and with higher cell viability than controls. Corroborating with the hypothesis that DPSCs and SHEDS tend to increase the activity of antioxidant enzymes, thus acting in the management of oxidative stress and raising the possibility of using these pulp stem cells in cell therapy for the treatment of oxidative stress-related diseases.

4. Conclusion

We can conclude that, generally, the cell viability of DPSCs and SHEDs submitted to H_2O_2 is dose and time dependent. SHEDs P3, seems to have the capacity to adept to a oxidative stress environment, showing increase in cell viability when summited to 50 µM of H_2O_2 This is follow by the increase of SOD activity. SHEDs P10 and DPSCs P3 and P10 showed lower capacity to increase cell viability when submitted an oxidative stress environment. To the best of our knowledge this is the first time that DPSCs and SHEDs are evaluated for their tolerance to an oxidative stress environment. However it is important to highlight the limitations of this study, since it is a pilot study and more analysis will be conducted in order to investigate SOD activity in DPSCs P3 and P10 and SHEDs P3, as well as other antioxidant enzymes.

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7 Considerações finais

Observa-se que ao longo dos últimos 15 anos, muitos estudos tem sido realizados utilizando DPSCs. Entretanto, é recorrente a falta de padronização das técnicas utilizadas para a manutenção dos elementos dentais, bem como das técnicas de isolamento celular, propriamente ditas. Dada à relevância e o impacto que essas variáveis possuem em relação às características das células cultivadas *in vitro*, é importante reforçar que os pesquisadores carecem estabelecer protocolos padrão para promover isolamento de modo uniforme. Num segundo momento, no que tange os ensaios laboratoriais, conclui-se que DPSCs e as SHEDs possuem capacidade de adaptação a um insulto com H2O2, sendo concentração-dependente. E embora se saiba da necessidade de aprofundar os conhecimentos nessa área, sugere-se que DPSCs na passagem 3 e 10 e SHEDs na passagem 10, futuramente, podem ser consideradas como uma possível ferramenta terapêutica.

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Apêndices

Apêndice A – Termo de Consentimento Livre e Esclarecido

Universidade Federal de Pelotas

Faculdade de Odontologia

Autorização para Pesquisa e Execução de Tratamento

Projeto: Isolamento e caracterização de células-tronco de tecido pulpar dental.

Responsável: Prof. Flávio Fernando Demarco

NOME DO PACIENTE:

FICHA N.°: _____

Por este instrumento que atende às exigências legais, o (a) senhor (a) _____, portador (a) da cédula de identidade n° _____, SSP/_____, após leitura minuciosa da CARTA DE INFORMAÇÃO AO PACIENTE, devidamente explicada pelo (s) profissional (is) em seus mínimos detalhes, ciente dos serviços e procedimentos aos quais será submetido, não restando quaisquer dúvidas a respeito do lido e do explicado, firma seu CONSETIMENTO LIVRE E ESCLARECIDO, em concordância em participar da pesquisa proposta no que lhe é cabível, conforme a CARTA DE INFORMAÇÃO AO PACIENTE.

Fica claro que o paciente ou seu representante legal pode, a qualquer momento, retirar seu CONSENTIMENTO LIVRE E ESCLARECIDO, sem ser prejudicado no tratamento, e deixar de participar do estudo alvo da pesquisa e ciente que todo trabalho realizado torna-se informação confidencial guardada por força do sigilo profissional (Art. 9° do Código de Ética odontológica).

Por estarem entendidos e conformados, assinam o presente termo.

Pelotas, _____ de _____ de 201_:

_____ _____

Assinatura do paciente Responsável pelo estudo

Apêndice B – Carta de Informação ao Paciente

UNIVERSIDADE FEDERAL DE PELOTAS
FACULDADE DE ODONTOLOGIA

Carta de Informação ao Paciente

Este estudo englobará pacientes que utilizam os serviços do setor de Cirurgia Buco-maxilofacial e Clínica Infantil da Faculdade de Odontologia, e que por necessidade clínicas e terapêuticas necessitarão remover os terceiros molares e molares decíduos hígidos.

Uma vez indicado a necessidade de remoção do(s) dente(s) (terceiro molar) pelos professores responsáveis do setor de Cirurgia Buco-maxilofacial e Clínica Infantil da Faculdade de Odontologia, o(s) mesmo(s) será(ão) removido(s) e armazenado(s) em um meio de cultivo até o seu processamento no laboratório. As células no interior do dente (tecido pulpar) serão então isoladas, cultivadas e caracterizadas por uma série de etapas laboratoriais. Este trabalho tem por finalidade adquirir estas células para que possamos estudar novas formas de tratamento que possibilitem curar o tecido no interior do dente, que por algum motivo tenha sofrido algum dano. É importante, porém ressaltar que o paciente não terá um benefício imediatos, pois este projeto visa resultados a longo prazo em função da sua complexidade.

Desta forma, estando informado do estudo que será realizado, dou pleno consentimento à Faculdade de Odontologia de Pelotas para que, por intermédio de seus professores, alunos de pós-graduação e graduação devidamente autorizados, utilizem o material biológico coletado, de acordo com os conhecimentos científicos e de forma ética.

Concordo também, que a documentação relativa ao estudo deverá ser arquivada na Faculdade de Odontologia e mantida sob a guarda dos autores do projeto de pesquisa, que se comprometem a manter sigilo dos dados coletados, não relacionando as células obtidas com qualquer dado dos pacientes, garantir que as informações geradas pelos resultados desse trabalho serão divulgadas apenas com finalidade cientifica e de ensino, como na redação de artigos e confecção de aulas, preservando-se, totalmente, o anonimato dos pacientes. Assim dou aos autores deste projeto de pesquisa, plenos direitos de uso, para fins de ensino e divulgação, respeitando os respectivos códigos de ética.

Contato em caso de dúvidas: Faculdade de Odontologia, rua Gonçalves Chaves, 457 CEP: 96015-560 - Pelotas - RS
Fones: (53) 32256741/ (53) 32224162/ (53) 32226690/ (53) 32224439/ (53) 32224305.

Pelotas, ____ de _____ de 201__.

_____ Documento: _____

Assinatura do paciente N.° _____

Apêndice C – Nota da Dissertação

Isolamento de células-tronco da polpa dental e análise da capacidade de combate ao estresse oxidativo

Dental pulp stem cells isolation and analysis of combat capability to oxidative stress

A presente dissertação de mestrado desenvolveu duas revisões sistemáticas da literatura, que abordam aspectos fundamentais sobre o método de obtenção de células tronco da polpa dental. Além disso, o referido trabalho investigou laboratorialmente a capacidade de adaptação e manejo de células-tronco da polpa dental quando em contato com um agente facilitador para geração de um ambiente com altos níveis de estresse oxidativo, H_2O_2. Nesse sentido foi possível observar uma inconsistência nos dados a respeito do manejo dental previamente ao isolamento celular propriamente dito, bem como de diversos aspectos que cercam essa prática. No que tange o ensaio laboratorial, os resultados parecem terapeuticamente promissores, revelando que células-tronco de dentes permanentes e decíduos, parecem reagir de forma a modular o estresse oxidativo.

Campo da pesquisa:

Candidato: Camila Perelló Ferrúa, Cirurgiã-dentista pela Universidade Federal de Pelotas (2013)

Data da defesa e horário: 09/02/2015, ás 10 horas.

Local: Auditório do Programa de Pós-graduação em Odontologia da Universidade Federal de Pelotas. 5º andar da Faculdade de Odontologia de Pelotas. Rua Gonçalves Chaves, 457.

Membros da banca: Prof[a]. Dr[a]. Sandra Beatriz Chaves Tarquínio, Prof[a]. Dr[a]. Rachel Krolow Santos Silva Bast e Prof[a]. Dr[a]. Adriana Fernandes da Silva. (Suplente)

Orientador: Prof[a]. Dr[a]. Fernanda Nedel
Co-orientadores: Prof. Dr. Flávio Fernando Demarco e Prof[a]. Dr[a]. Gabriele Cordenonzi Ghisleni

Informação de contato: Camila Perelló Ferrúa. camila_perello@hotmail.com
Endereço para contato: Rua Gonçalves Chaves, 457 – Núcleo de Biologia Celular e Tecidual.

Apêndice D – Súmula do currículo do candidato

Súmula do currículo[2]

Camila Perelló Ferrúa, nasceu em 22 de fevereiro de 1989, em Pelotas, Rio Grande do Sul. Completou o ensino fundamental e médio em Escola privada na mesma cidade. No ano de 2008 ingressou na Faculdade de Odontologia da Universidade Federal de Pelotas (UFPel), tendo sido graduada cirugiã-dentista em 2013/1. Durante o período de graduação foi bolsista de iniciação científica sob orientação do professor Flávio Fernando Demarco Em 2013/2, foi aluna especial do Programa de Pós-Graduação em Odontologia, pela Universidade Federal de Pelotas. No ano seguinte ingressou no mestrado do referido programa, como aluna regular, na área de concentração Dentística, sob orientação do Profª. Drª. Fernanda Nedel.. Durante o período de mestrado foi bolsista da Comissão de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) e desenvolvou trabalhos na biologia celular e engenharia tecidual.

Publicações:

ALEVES, ALESSANDRO MENNA ; FERRÚA, CAMILA PERELLÓ ; CARVALHO, PEDRO HENRIQUE DE AZAMBUJA ; TARQUINIO, SANDRA BEATRIZ CHAVES ; ETGES, ADRIANA; NEDEL, FERNANDA; LUND, RAFAEL GUERRA. Comparison of two storage conditions of *Candida albicans* for DNA extraction and analysis. V.9, p.1849-1852, july 2015.

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Anexos

Carta de aprovação do Comitê de Ética em Pesquisa

MINISTÉRIO DA EDUCAÇÃO

UNIVERSIDADE FEDERAL DE PELOTAS

FACULDADE DE ODONTOLOGIA

COMITÊ DE ÉTICA E PESQUISA

PELOTAS, 15 de julho de 2013

PARECER Nº 38 /2013

O projeto de pesquisa intitulado **"Isolamento e caracterização de células–tronco de tecido pulpar dental".** está constituído de forma adequada, cumprindo, nas suas plenitudes preceitos éticos estabelecidos por este Comitê e pela legislação vigente, recebendo, portanto, PARECER APROVADO.

Prof. Dr. Renato Fabrício de Andrade Waldemarin

Coordenador do CEP- FOP/UFPel

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