Dextrose solution used for prolotherapy decreases cell viability and increases gene expressions of angiogenic and apopitotic factors

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ABSTRACT

Objectives: Hypertonic dextrose injection in prolotherapy is an injection-based treatment used in chronic musculoskeletal conditions. Dextrose prolotherapy raises growth factor levels and enhances tissue repair, reduces musculoskeletal pain. Despite of uses for many years, the effect of dextrose solution on cellular and molecular base is not fully clear. Here, the roles of dextrose solutions was tried to find out in different concentrations on human fibroblasts in vitro. Gene expression alterations were analyzed in uses of dextrose solutions on growth and apoptotic factors.

Methods: The effects of dextrose solution (1%, 5%, 10%-low doses, 15%, 20% and 25%-high doses) were evaluated in vitro by using human fibroblast culture. In each condition total RNA extraction and cDNA synthesis were performed. The gene expression levels of angiogenic and apoptotic factors were analyzed by using real-time polymerase chain reaction. The gene expression results of growth and apoptotic factors were correlated with control results.

Results: Results; Dextrose solutions were affected the viability of fibroblast cells in culture flask in high concentrations. In high doses dextrose concentrations, up to 80% of fibroblasts were died because of toxic conditions. Viable fibroblast cell ratios were decreased proportionally due to the dextrose concentration. Low dextrose concentrations increased gene expressions in angiogenic (VEGFA, PDGFA, PDGFB, IGF1) and in apopitotic factors (CASP3 and CASP8) in fibroblasts.

Conclusions: Conclusion; Dextrose solution in high concentrations, decreases viable cell ratios on adult fibroblast cell line. Dextrose solutions in proper concentrations increase the gene expressions of angiagenic and apopitotic factors on viable cells in adult fibroblast cell culture.

Introduction

Prolotherapy (Short for “proliferation therapy”) is a nonsurgical treatment which stimulates healing. It is also known as nonsurgical ligament and tendon reconstruction, or regenerative injection therapy. Prolotherapy works by stimulating the body’s own natural healing mechanisms to repair injured musculoskeletal tissue. Generally osmotic agents for example dextrose solution are used in clinical usage (1, 2). The aim of prolotheraphy is to create a controlled acute inflammation by injecting the proliferative solutions repeatedly to area which have chronic problems. The proliferative solutions are believed to create local irritation, inflammation and tissue regeneration. As a result they increase the strength of the damaged ligament, tendon and intra articular structures (3). Dextrose solutions participate in healing process as a starter and a regulator. Platelets, macrophages and fibroblasts are attracted to the injection area during inflammation stage. Many growth factors such as platelet derived growth factor (PDGF), transforming growth factor (TGF), insulin like growth factor (IGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are secreted by platelets, macrophages and fibroblasts for healing process (4, 5, 6). Despite of these findings the role of dextrose on fibroblast is not fully clear. In this manuscript, the gene alterations were analyzed which have roles on cell growth and apopitosis on fibroblasts with use of dextrose solution.

Dextrose solutions (in 1%, 5%, 10%, 15%, 20% and 25% concentrations) were applied on adult human fibroblasts in vitro culture condition. Cell proliferation assay was used for finding the cell deaths in cell culture. Up to 80% fibroblast cells died in high dextrose concentrations (15%, 20% and 25%). The deaths of cells were visualized on inverted microscope. In low dextrose concentrations (1%, 5% and 10%), viable cell ratio was above 80%. The gene expression analysis were performed on selected genes which have roles on angiogenesis and apopitosis [VEGFA (Vascular Endothelial Growth Factor A- OMIM

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NO: 192240), PDGFA (Platelet Derived Growth Factor Subunit A- OMIM NO: 173430), PDGFB (Platelet Derived Growth Factor Subunit B- OMIM NO: 190040), IGF1 (Insulin Like Growth Factor 1- OMIM NO: 147440), CASP3 (Caspase 3- OMIM NO: 600636) and CASP8 (Caspase 8- OMIM NO: 601763), CASP10 (Caspase 10- OMIM NO: 601762), BAX (BCL2 Associated X, Apoptosis Regulator – OMIM NO: 600040) and BCL2L1 (BCL2 Like 1- OMIM NO: 600039)]. We tried to find the possible roles of high and low dextrose concentrations on cell viability and angiogenesis capacity in prolotherapy applications. Also affected mechanisms in prolotherapy applications were tried to explain in cellular and molecular genetics base.

Methods

Cell Culture

This study was designed due to Gülhane Military Medical Academy, ethic council decision (06/01/2015- Meeting No; 01). Adult human fibroblasts (ATCC PCS-201-012) were cultured and incubated in RPMI-8226 1640 (Sigma-Aldrich-R8758) including 10 % (v/v) FBS (BiochromAG, Germany) and 1% (v/v) gentamycine (Biological Industries, Israel) at 37 ° C in 5% CO_2 Heraus incubator (Hanau, Germany). We arranged seven cell culture groups; one for control, six for prolotherapy solutions (1%, 5%, 10%, 15%, 20% and 25% dextrose solutions).

Preparing prolotheraphy solutions

Dextrose (Sigma D9434) was used in the preparation of dextrose solution in 1%, 5%, 10%, 15%, 20% and 25% concentrations.

Application of solutions

In control group, only RPMI-1640 (Sigma R8758) as a cell culture medium was used. For study groups, 2 ml of dextrose solution in each was used for each culture flask. The cells were examined immediately after the applications and 24th hour later. The changes in cell morphology were noted. The cells were photographed for morphological analyses.

Cell viability assay; Trypan blue (Sigma Aldrich Co. 302643) as a stain was used in procedures for viable cell counting. Trypan blue was diluted at 0.8 mM in PBS. It was mixed with the cells 1:1. In this method, viable and non-viable cells were counted on hemocytometer (7).

RNA isolation and cDNA synthesis

All of the culture flasks were harvested by Trypsin-EDTA solution (Sigma Aldrich Cat No: T4049) 24 hours later. RNA isolation procedure was done on the harvested cells according to manufacturer's protocol (NucleoSpin RNA-Machenery Nagel). For cDNA synthesis, Real Time PCR (RT- PCR) was used as 42° for 60 minutes; 70° for 5 minutes according to the manufacturer' s protocol (Fermentas, Revert Aid cDNA synthesis kit). c-DNA' s were controlled on 2% agarose gel.

Reverse transcription-polymerase chain reaction-RT PCR;

The gene expression levels of VEGFA, PDGFA, PDGFB, and IGF1 for healing process and CASP2, CASP3, CASP8, CASP10, BAX and BCL2L1 for apoptosis were analyzed. The primer lists were found in primer bank (http://pga.mgh.harvard.edu/cgi-bin/primerbank). The obtained cDNAs from all groups were used as template for RT-PCR. RT-PCR conditions: 95˚C° 10'', 56˚ 15'', 72˚C 15''- 45 cycle (Roche Light Cycler 1.5). For internal control β-Actin gene was used [beta-actin housekeeping gene (5'-GTC CCT CAC CCT CCC AAA AG-3' (forward) and 5'-GCT GCC TCA ACA CCT CAA CCC-3' (reverse)]. Each RT-PCR reaction is performed in 20 µl (10 µl 2x SYBR, 5 µl c DNA, 0.5 µl primer, 3 µl d H_2O). Each sample was studied for three times for proper statistical results. Results were analyzed by "Roche Light Cycler1.5 software".

Statistical Analyses

Mean values and standard deviations were obtained, in cell viability assay. For evaluating the results of RT-PCR analyses, Student T test was used in the comparison of groups.

Results

1. In all cell culture groups, cell viability ratio was found over 90% before dextrose treatment. In high dextrose concentrations (15%, 20% and 25%), cell death was observed almost most of cells within 24 hours. In inverted microscope, we observed that the cells separated from the flask surface. Dysmorphic fibroblasts were seen almost in all areas that visualized . Up to 80% of cells were found as non-viable in this series. Culture flasks, viability ratio lower than %20 were eliminated in the molecular analyses part of the study.
2. In low dextrose concentrations (1%, 5% and 10%), the cells remained intact in flasks. Dysmorphic fibroblasts were few in all areas that visualized. The comparison with the control group in cell dysmorphology and cell viability, similar results were obtained. The viable cell ratios were over %93, %91 and %81 in 1%, 5% and 10% dextrose groups respectively. Decreased cell viability ratios were noted with high dextrose concentrations in our experiments. We did the molecular gene expression analyses of selected genes on the low dextrose concentration group.
3. In control group. the gene expression analyses results were found as 0.005±0.02 for VGFA; 0.020±0.01 for PDGFA; 0.007±0.03 for PDGFB; 0.012±0.01 for IGF1 in angiogenic factor group (Table-1).
4. VGFA gene expression analysis revealed that increased expression levels were found in low dextrose concentrations. In PDGFA/PDGFB and IGF1 genes, expression analysis revealed increased expression levels in low dextrose concentrations (Table-1). All the results in VGFA, PDGFA/PDGFB and IGF1 genes were statistically significant (Table-1). So, the genes participate in angiogenic factors revealed increased expression levels with the dextrose solutions, in our study.
5. In control group. the gene expression analyses results were found as 0.002 for CASP 2; 0.009 for CASP3; 0.009 for CASP8; 0.022 for CASP10; 0.009 for BAX; 0.002 for BCL2L1 in apopitotic factor group (Table-1).

CASP2, CASP10, BAX and BCL2L1 gene expression analysis revealed that no expression difference was found in low dextrose concentrations. All the results in CASP2, CASP10, BAX and BCL2L1 genes were statistically insignificant (Table-1). CASP3 and CASP8 genes were found as high due to control in low dose dextrose application in test tube. So, the genes participate in apopitotic factors revealed increased expression levels with the dextrose solutions, in our study (Table-1).

Table-1. The gene expression results of control and study groups

		Control	1% Dextrose Solution	P	5% Dextrose Solution	P	10% Dextrose Solution	P
Angiogenic Factors	***VEGFA***	0.005±0.02	0.020±0.01	<0.05	0.080±0.04	<0.05	0.090±0.09	<0.05
	PDGFA	0.020±0.01	0.100±0.04	<0.05	0.120±0.11	<0.05	1.710±0.09	<0.05
	PDGFB	0.007±0.03	0.005±0.01	<0.05	0.003±0.01	<0.05	0.040±0.01	<0.05
	IGF1	0.012±0.01	0.100±0.02	<0.05	0.080±0.03	<0.05	2.340±0.09	<0.05
Apopitotic Factors	***CASP 2***	0.002±0.02	0.003±0.02	≥0.05	0.004±0.02	≥0.05	0.050±0.05	≥0.05
	CASP3	0.009±0.13	0.090±0.11	<0.05	0.140±0.09	<0.05	0.750±0.12	<0.05
	CASP8	0.009±0.01	0.070±0.03	<0.05	0.070±0.08	<0.05	2.100±0.07	<0.05
	CASP10	0.022±0.11	0.020±0.09	≥0.05	0.022±0.07	≥0.05	0.200±0.06	≥0.05
	BAX	0.009±0.02	0.070±0.03	≥0.05	0.170±0.04	≥0.05	2.100±0.02	≥0.05
	BCL2L1	0.002±0.13	0.090±0.04	≥0.05	0.140±0.05	≥0.05	0.750±0.02	≥0.05

Discussion

Prolotherapy involves injecting an irritant, such as hyperosmolar dextrose, into a joint space, ligament or tendon with the ultimate goal of alleviating pain (8). The mechanism is not fully understood but there is evidence that an inflammatory response causes growth factor release, which stimulates collagen deposition and tissue repair (9). There is good evidence that prolotherapy is an effective tendinopathy treatment, that significantly reduces neovascularisation which often correlates with clinical improvement (10). There is conflicting evidence regarding the efficacy of prolotherapy injections in reducing pain and disability in patients with chronic musculoskeletal pain (11). Although there are many clinical studies about prolotherapy solutions, there are a few in vitro studies among the effects of these solutions. So we aimed to find out the possible effects of these solutions on the cells in molecular level, studying in vitro.

Cell death is the event of a biological cell ceasing to carry out its functions. This may be the result of the natural process of old cells dying and being replaced by new ones, or may result from such factors as disease, localized injury, or toxic substances (12). Here, cell death observed in our fibroblast culture was probably occurred as a result of intoxication. Despite of these findings, the type of cell death (apopitosis or necrosis) is not clear. Programmed cell death (apopitosis) is cell death mediated by an intracellular program. Apoptosis or Type I cell-death, and autophagy or Type II cell-death are both forms of programmed cell death, while necrosis is a non-physiological process that occurs as a result of infection or injury. Necrosis is cell death caused by external factors such as trauma or infection, and occurs in several different forms (13). Apoptosis is the process of programmed cell death that may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. These changes include blebs, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (14). To investigate the effect of apoptosis on cell morphology, we applied high and low doses of dextrose on fibroblast cell culture. Regular apoptotic changes and cell death were noted in fibroblast cells treated with high concentrations of dextrose. We observed that morphology of the cells changed after dextrose treatment in cell viability assay analyses (15). Cell proliferation and cell viability ratios were decreased.

There are few articles about hyperosmotic application of glucose and cell behavior in literature. Barros et al. reported that hyperosmotic shock application with 2-deoxy-D-glucose increased the uptake of sugars in epithelial cells. Hyperosmotic shock induced both activation and translocation of glucose transporters (GLUT1 and GLUT4) in mammalian cells (16). Despite of these findings, another manuscript mentioned that GLUT1 can be activated in fibroblast cells by glucose deprivation. The authors found that alkaline pH activates the transport activity of GLUT1 in fibroblast cells (17). Literature findings presented that a lot of study are needed about the mechanisms affected in hyperosmolar treatment in a cell.

Excessive intraperitoneal absorption of glucose during peritoneal dialysis has both local cytotoxic and systemic metabolic effects “in vivo” (18). Eryptosis (the suicidal erythrocyte death) characterized by cell shrinkage and cell membrane scrambling, is stimulated by Ca(2+) entry through Ca(2+)-permeable, PGE2-activated caution channels, by ceramide, caspases, calpain, complement, hyperosmotic shock by hyperosmolar dextrose, energy depletion, oxidative stress, and deranged activity of several kinases (e.g. AMPK, GK, PAK2, CK1α, JAK3, PKC, p38-MAPK). So, hyperosmotic shock by using hyperosmolar dextrose is a reason of cell death (19). Similar results were obtained in our study. Dextrose intoxication caused cell death in our culture flasks. With these findings, we can’t distinguish the type of cell death (apopitosis or necrosis). For finding the type of cell death, apopitotic factors were studied in our panel. In CSAP3 and CASP8 genes, statistically significant increases were observed in our panel. As known, caspases are known as the principal mediators of the apoptotic cell death response (20). In autophagy, the modulator roles of caspases have only begun to be described. In contrast to apoptosis, autophagy promotes cell survival by providing energy and nutrients throu-

gh the degradation of lysosome. So, autophagy and apoptosis regulate each other in cell survival and death using autophagy related proteins and caspases (21, 22). In apopitosis, two activation mechanisms are known (intrinsic and extrinsic pathway) (23). At the beginning of intrinsic pathway, intracellular signals are generated when cells are stressed. This mechanism causes apopitosis using mitochondrial proteins. The extrinsic pathway is activated by extracellular ligands binding to cell-surface death receptors, which leads to the formation of the death-inducing signaling complex. CASP2, CASP3, CASP8, CASP10, BAX and BCL2L1 factors studied in our test panel are common factors used in these two apopitotic pathways (24). The gene expression analysis revealed that CASP3 and CASP8 had higher gene expressions than control (Table-1). High dextrose concentration urges the cells in culture flasks to apopitosis probably using CASP3 and CASP8 genes (25).

VEGF is a signal protein produced by cells that stimulates the formation of blood vessels (26). PDGF is one of numerous growth factors that regulate cell growth and division. In particular, PDGF plays a significant role in blood vessel formation, the growth of blood vessels (27). IGF-1 is a hormone similar in molecular structure to insulin. It plays an important role in childhood growth and continues to have anabolic effects in adults (28). VEGF, PDGF and IGF gene expressions were found higher in uses of low dose dextrose than in control (Table-1). These genes are active in angiogenesis and healing process (29, 30). Due to our analyses, dextrose increases angiogenesis and apopitosis via VEGF, PDGF, IGF, CASP3 and CASP8 genes. It causes wound healing in prolotherapy.

Angiogenesis means the development of new blood vessels out of existing ones, due to triger factors such as ischemic diseases, hypoxia, injuries and tumour growth with the secretion of some angiogenic factors (31). The chorioallantoic membrane assay (CAM) is an ex vivo model system for the study of vascular anomalies and neoangiogenesis (31, 32). Larger et al. evaluated the direct effect of hyperglycemia on angiogenesis in the chicken CAM assay. They found that hyperglycemia impairs angiogenesis through induction of apoptosis and decreased proliferation of endothelial cells (33). Similar results were observed in our study. Hyperosmotic dextrose treatment stimulated cell death via induction of apoptosis. It also decreased cell proliferation. In CAM assay, Larger et al. found that hyperglicemia affected angiogenesis without altering the expression level of vascular growth factors (33). We observed in our study that hypergilisemia induced angiogenesis via VEGF, PDGF, IGF on certain doses. These findings seem an incompatibility with the literature finding. Angiogenesis generally occurs on certain growth factors including VEGF, PDGF, IGF (34, 35). Probably the suitable hyperosmotic dextrose doses applied in our study presented such kind of result on growth factors. On the other hand CAM assay used in Larger et al.' experiment is an "in vivo" model (31). The methodology applied in our study is an "in vitro" model. The methodology chosen in our experiments may cause such a kind of incompatibility.

As a result, dextrose solutions in low concentrations enhance local wound healing process in certain areas in prolotherapy. In application of high dose dextrose concentrations, local cell deaths may cause enhanced stimulations which are responsible for the reaction of healing process.

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Conflict of Interest

The author declared he does not have anything to disclose regarding conflict of interest with respect to this manuscript.

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