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Effects of lipid and glucose on in vitro nodule formation and calcification by sheep aortic smooth muscle cells

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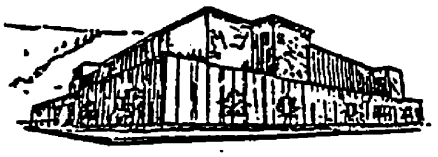

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Effects of Lipid and Glucose on *In Vitro* Nodule Formation and Calcification by Sheep Aortic Smooth Muscle Cells

by

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Effects of Lipid and Glucose on *In Vitro* Nodule Formation and Calcification by Sheep Aortic Smooth Muscle Cells.

Director: George Card

Vascular calcification, closely associated with atherosclerosis and diabetes mellitus, contributes significantly to patient mortality. Diabetes mellitus is characterized by hyperglycemia and hyperlipidemia. Several *in vitro* studies have explored the influence of lipids, particularly 25-hydroxycholesterol, on increased calcification and possible increased atherosclerotic lesion formation. However, most of these studies were conducted at hyperglycemic glucose levels, used an oxidized form of cholesterol and did not explore a possible interdependence between glucose and lipid on these effects.

Studies to evaluate the effects of lipid and glucose on smooth muscle cells using an ovine *in vitro* model were performed in the absence and presence of additional cholesterol (48.7 mg/dL, 18.8 mg/dL) or triglyceride (173 mg/dL) at both 100 mg/dL (low) and 450 mg/dL (high) glucose. Nodules, multi-cellular aggregates that calcify *in vitro*, were counted and calcium deposition was measured.

Cholesterol increased the number of nodules formed, dose-dependently, and the rate of nodule formation at both low and high glucose. Triglyceride had a marginal effect. Cholesterol did not cause significant changes in calcium deposition at either low or high glucose. The increased nodule formation and rate of nodule formation observed at high cholesterol was much greater at low glucose, suggesting that glucose influenced the lipid effects on smooth muscle cell nodule formation.

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List of Abbreviations

25-OHC	25-hydroxycholesterol
AGE	advanced glycation endproducts
A-HP	protein A-horseradish peroxidase
ALP	alkaline phosphatase
ASMC	aortic smooth muscle cell
BASMC	bovine aortic smooth muscle cell
β-GP	beta-glycerophosphate
BMP-2	bone morphogenetic protein 2
BrdU	5-bromo-2'-deoxyuridine-5'-phosphate
Ca^{45}	calcium 45
CVC	calcifying vascular cell
CVvC	calcifying valvular cell
DM	diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DMF	N, N-dimethyl-formamide
FBS	fetal bovine serum
GTPases	guanosine triphosphatases
HA	hydroxyapatite
HCl	hydrogen chloride
HMG-CoA	β-hydroxy-β-methylglutaryl-coenzyme A
IACUC	Institutional Animal Care and Use Committee
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
OPN	osteopontin
ox-LDL	oxidized low density lipoprotein
P	statistical variance
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
SASMC	sheep aortic smooth muscle cell
SDS	sodium dodecylsulfate
SMC	smooth muscle cell
TGF	transforming growth factor
TGF-β1	transforming growth factor beta 1
UV	ultra-violet
VASMC	vascular aortic smooth muscle cell
VSMC	vascular smooth muscle cell
VWF	von Willebrand factor

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1.0 INTRODUCTION

1.1 Introduction

In all tissues except bone, cells actively sequester calcium intracellularly from ubiquitous phosphate thereby preventing mineralization, the precipitation of calcium-phosphate crystals. Dystrophic calcification is the pathological mineralization within soft tissues, often in response to injury. As a common feature in many pathological conditions in the vasculature, dystrophic calcification contributes significantly to patient morbidity and mortality. Dystrophic mineralization in native and bioprosthetic valves is the leading cause of stenotic (regurgitant) failure, which can cause arrhythmia, stroke and heart failure [1, 2]. Arterial calcification, used as a predictor of future adverse cardiovascular events and death, has been positively correlated with atherosclerosis, diabetes, heart disease, cardiac valve disease, coronary artery disease and with increased risk of myocardial infarction, unstable angina, dissection after angioplasty and ischemic episodes in peripheral vascular disease [1-5]. There is a nearly invariable clinical association between vascular calcification and atherosclerosis, and advanced atherosclerosis is frequently associated with diabetes [3]. Most atherosclerotic plaque ruptures that lead to sudden death are associated with calcification [6].

Despite a frequent role in pathogenesis, vascular dystrophic calcification is an undefined process with many intricate molecular mechanisms yet to be elucidated. Calcification has been viewed as a passive process, a direct consequence of cell injury and/or death dependent only on concentrations of phosphate and calcium that form a precipitate when mixed [7, 8]. Based on observed similarities between cells and factors involved in both calcified atherosclerotic plaques and normal bone formation, Demer and

others began in the mid-1990s to rethink the overall process of dystrophic calcification, suggesting that rather than being a passive process, vascular dystrophic calcification is an active and regulated process [3, 6, 7, 9]. Both physiological (bone) and dystrophic (soft tissue) calcification involve collagen-associated crystal deposition and initiation of mineralization in the form of hydroxyapatite (HA) crystals within matrix vesicles, which are extracellular blebs resembling apoptotic bodies [4-6, 10]. Thus apoptosis, regulated cell death, may be involved [10]. A sub-population of cells from the artery wall with characteristics of osteoblasts, the normal bone-building cells, was shown to spontaneously form mineralized nodules in cell culture, suggesting the cells necessary for vascular calcification reside in the vasculature [7, 9, 11-13]. Such cells are thought to arise through differentiation of progenitor cell types, such as pericytes, or smooth muscle cells (SMCs), both of which have been demonstrated to undergo phenotypic modulation. This supports the notion that there are active regulatory mechanisms shared by bone and dystrophic calcification [6]. Alkaline phosphatase (ALP), an enzyme that frees phosphate, and osteopontin (OPN), an HA-binding protein, may be key players, as these have been detected *in vivo* in both atherosclerotic plaques and bone, apparently influenced by several regulating factors common to these sites [2, 4, 5, 9]. Both ALP and OPN have also been found in calcified nodules within vascular cell cultures[14]. The regulatory involvement of vitamin D, a structurally similar derivative of cholesterol, seems a key to unraveling the mysterious correlation between osteoporosis and vascular dystrophic calcification, where bone is being both degraded and produced at different locations within the same individual [6,7,14].

Diabetes mellitus (DM), a pathological condition associated with increased atherosclerosis, may increase dystrophic calcification through serum factors found at abnormal levels acting both indirectly and directly on vascular wall cells. Glucose, insulin, triglycerides and cholesterol are all serum factors that are found at elevated levels during DM. The mechanisms active in DM may involve oxidized forms of cholesterol acting through vitamin D receptors, which are found on smooth muscle cells [6,7,14].

To elucidate the role that serum factors play in vascular dystrophic calcification mechanisms and to evaluate potentially exacerbating or therapeutic factors, it is necessary to examine individual and combined effects of soluble agents in the serum. Diabetic states of high glucose alone or in conjunction with these other factors may influence the effect of therapeutic agents on vascular cells. The direct effect of statins, drugs widely used to lower serum cholesterol, on vascular smooth muscle cell calcification needs to be evaluated. The increased osteoblastic differentiation by bone cells in response to statins may also be observed in vascular cells, potentially leading to dystrophic calcification.

1.2 Background and Significance

Several *in vitro* studies have used subpopulations of cells found in the vasculature, including SMCs [1, 4, 15, 16], pericytes [3], calcifying vascular cells (CVCs) [6, 7, 13] and calcifying valvular cells (CVvCs) [11], in efforts to study factors influencing vascular dystrophic calcification and/or associated disease conditions such as atherosclerosis and osteoporosis [2, 7]. All these cell types have demonstrated calcification in cell culture, in the form of apatite crystals, the same mineral found *in vivo* in bone and atherosclerotic plaques. One existing theory is that the mentioned cell types may actually be the same basic vascular cell derived from SMCs or pericytes. In cell culture, each cell type mentioned formed spontaneous nodules that subsequently calcified and expressed osteoblastic differentiation markers, including ALP [1, 3, 9, 11, 12]. Calcium deposition apparently involves phenotypic modulation of vascular cells that differentiate toward osteoblasts, suggesting a regulated process [3].

Increasing the rates of *in vitro* calcification by adding increased amounts of the phosphate donor beta-glycerophosphate β-GP, initially published by Shioi et al, has become a standard, accelerated method enabling *in vitro* quantification of calcification in dystrophic calcification models [3, 4, 9]. Extracellular matrix produced *in vitro* by bovine aortic SMCs (BASMCs) accumulates apatitic (calcium phosphate) mineral after β-GP addition [1, 4, 9]. Apatite is deposited along and within collagen fibrils in the presence of matrix vesicles, correlating to calcified vascular tissues seen *in vivo*, particularly in atherosclerotic plaques [1, 4, 9]. The increased BASMC calcification by β-GP is inhibited by the ALP inhibitor levamisole [9]. Alkaline phosphatase (ALP), which uses β-GP as a

substrate, is required for calcification of osteoblast, cartilage and smooth muscle cell cultures in response to β-GP [1]. Rat bone marrow stromal cells and mouse clonal osteoblastic cell lines were shown to form calcified nodules and to express ALP [2,17]. Thus, alkaline phosphatase has become a common marker of osteoblastic differentiation [3, 5, 9, 11].

Interstitial cells cultured from both human and canine cardiac valves (CVvCs) undergo phenotypic change *in vitro*, developing osteoblastic-like characteristics including alkaline phosphatase (ALP) expression and formation of calcific nodules [11]. Mineralized nodules in cell culture and their association with extracellular bone-building cells suggests that the cells necessary for vascular calcification reside in the vasculature. Compared to human, canine CVvCs show decreased time for nodule formation, and nodules formed from both canine and human sources are morphologically indistinguishable, suggesting that an animal versus a human model may be preferable in terms of time and cost [11].

In vitro models demonstrate that the rate of calcification and of nodule formation can be modified by the addition of soluble factors to vascular cell media. Abnormal lipid levels and lipoprotein profiles are common in patients with diabetes mellitus [18]. The presence of 25-hydroxycholesterol (25-OHC) in atherosclerotic plaques and its postulated regulatory role in bone osteoblast differentiation and matrix production, led to the investigation of the lipid role in dystrophic calcification models [13]. The research into lipid involvement was further prompted by the inhibition of calcification of porcine valve tissue after selective lipid removal [19].

25-OHC increased the formation of nodules and the number of calcified (von Kossa stain positive) nodules by CVvCs [11, 13]. The oxidized lipids 25-OHC, oxidized-low density lipoprotein (ox-LDL) and 8-isoprostaglandin E_2 all increased calcification, mineralized nodule formation and ALP activity by CVCs [5-7, 9]. These oxidized lipids had the opposite effect on bone pro-osteoblastic cells, suggesting osteoblast-like cells of the vasculature may respond differently than osteoblasts in bone to identical regulatory soluble factors [5-7]. The active form of vitamin D_3, an oxidized derivative of cholesterol, and dexamethasone, a synthetic glucocorticoid that stimulates osteoblasts *in vitro*, both increased calcification, ALP activity and OPN expression by BVSMCs [4, 14]. OPN alone decreased BVSMC calcification, with no change in ALP activity or phosphorus levels, and is thought to bind apatite crystals to slow calcification [1].

Hyperglycemia and hyperinsulinemia, both conditions found during diabetes mellitus, have a positive and additive effect on human arterial SMC proliferation [16]. Hyperglycemic conditions have been shown to influence VSMC receptor expression [20]. Shioi *et al* commented that insulin might affect calcification regardless of b-GP addition [9]. Advanced glycation endproducts (AGEs), formed at an accelerated rate during diabetes mellitus, are non-enzymatically glycated protein derivatives that are found in atherosclerotic plaques [3]. AGEs added to bovine pericytes increased the number of calcified nodules and calcium deposition by approximately the same degree (4x), suggesting nodule count may be a valid endpoint in evaluating calcification [3]. The abnormally elevated serum components found during diabetes mellitus have been shown

to modulate vascular cells, however, their direct effect on dystrophic calcification by vascular smooth muscle cells remains unknown.

Statins, competitive inhibitors of the rate-limiting enzyme (HMG-CoA reductase) of the mevalonate pathway, are currently used to lower levels of serum cholesterol [21, 22]. Mevalonate is a precursor of cholesterol and a variety of isoprenoid-containing compounds. Statins were shown by Mundy *et al* to increase the number of osteoclasts, the amount of new bone formed and the expression of bone morphogenetic protein 2 (BMP-2) in mouse skull bone cultures [21, 22]. The hypothesis is that inhibition of the mevalonate pathway by statins prevents prenylation and function of small GTPases, allowing increased BMP-2 expression, causing osteoblast differentiation, proliferation and enhanced bone formation [21, 22]. The direct effect of statins on vascular cell dystrophic calcification, as yet unknown, may increase osteoblast-like characteristics of these cells and thereby contribute to mineralization. The complexities of vascular dystrophic calcification require careful evaluation of multiple factors that may influence the overall outcome of therapeutic agents.

Dystrophic calcification appears to be a regulated process, which can be represented and explored using an *in vitro* model in which vascular (smooth muscle) cells form multicellular nodules that subsequently calcify.

1.3 Overall Objective and Hypotheses

The overall objective of this research was to create an *in vitro* model capable of evaluating the effect of factors on vascular dystrophic calcification during diabetic and non-diabetic states. The hypothesis was that blood plasma components found at abnormal levels *in vivo* during diabetes mellitus, a diseased state linked to increased rates and/or risk of dystrophic calcification, would demonstrate *in vitro* changes in the rates of nodule formation and calcification, thus providing insight into the role of these components in the pathogenesis of dystrophic calcification *in vivo*.

1.31 Specific Aims

The following specific aims were designed to address the objectives.

Specific Aim 1: Establish the *in vitro* model and characterize the nodules.

Specific Aim 2: Determine the correlation between number of nodules and amount of calcium in culture and evaluate whether this relationship establishes nodule count as a valid endpoint for measuring dystrophic calcification.

Specific Aim 3: Determine the influence on nodule formation and calcium assay at elevated levels of glucose (450 mg/dL) exerted by certain factors associated with increased dystrophic calcification, specifically hypercholesterolemia and hypertriglyceridemia.

Specific Aim 4: Determine the influence on nodule formation and calcium assay at baseline levels of glucose (100 mg/dL) exerted by certain factors associated with increased dystrophic calcification, specifically hypercholesterolemia and hypertriglyceridemia.

2.0 Materials and Methods

2.1 General Materials

Liquid Dulbecco's modified Eagle's medium (DMEM, both 450 mg/dL and 100 mg/dL glucose), fungizone, gentamicin, collagenase type III, liquid trypsin-EDTA, all tissue culture grade, were obtained from Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS) of the same lot number was acquired from Hyclone (Logan, UT). Calcium assay kit, D(+) glucose (tissue culture grade) and other chemicals (unless otherwise mentioned) were obtained from Sigma Chemical Co. (St. Louis, MO). All FBS used was heat inactivated (30 min at 56°C).

2.2 Cell Harvesting

Sections of sheep aorta were obtained from adult Targhee sheep undergoing experimental surgical procedures. Outer tissue was gently removed, and the pieces rinsed with DMEM. The margins were trimmed off and the remainder was cut into approximately 1 mm pieces, which were placed in 0.1% collagenase in phosphate buffered saline (PBS) and incubated for 1 hr at 37°C. Following incubation, the supernatant was removed and 10 mL of DMEM containing 10% FBS was added. The mixture was centrifuged at 1600 rpm for 5 min and the supernatant was again removed. The cells were resuspended in DMEM with 10% FBS, gentamicin and fungizone. These primary cultures of Targhee sheep SMCs were stored frozen at –80°C.

All animals were cared for in accordance with the "Principals of Laboratory Animal Care" formulated by the National Society of Medical Research, and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised 1996. The use of the animals for this research was also reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Montana.

2.3 Cell culture

Cells previously harvested from Targhee sheep aorta and frozen back were transferred to tissue culture plates and maintained in growth medium at 37°C in a humidified incubator containing 5% CO_2, with media exchanged every 2-3 days. Unless otherwise stated, growth medium was 500mL high glucose DMEM supplemented with 50mL FBS (10%), 0.5mL fungizone, 1.0mL gentamicin, 5mL L-glutamine. Low glucose growth medium refers to growth medium made with low (100 mg/dL) rather than high (450 mg/dL) glucose DMEM. Cultures capable of forming nodules within 7 days were selected for experiments and were used up to passage number 15. Prior to seeding cells, cell viability was quantified by Trypan blue exclusion. Cell counts were performed using a hemocytometer.

2.4 Experimental Design

Experiments were performed in 24-well tissue culture plates with cells seeded at a density of 2×10^5 cells/mL for 24-48 hours (or until confluence) in high glucose growth medium (described above) to allow establishment of an equivalent monolayer for all experiments. For experiments requiring histology, sterile tissue-culture treated coverslips (Fisher) were placed in each well prior to seeding. After confluence and every 2 days, the appropriate growth medium (DMEM with high 450 mg/dL or low 100 mg/dL glucose, 10% FBS, fungizone, gentamicin, L-glutamine) was added to wells, followed by addition of experimental reagents, at specified concentrations. SMCs were seeded for 24 hours in DMEM prior to the addition of lipid. Control wells without additional cholesterol contained approximately 3.9 mg/dL cholesterol, from the addition of 10% FBS used in regular DMEM medium. Cholesterol at high (additional 44.82 mg/dL, total 48.72 mg/dL) and low (additional 14.94 mg/dL, total 18.84 mg/dL) levels was applied within a commercial suspension high in cholesterol (Sigma Lipids – Cholesterol-rich). The additional high cholesterol was three times the additional low cholesterol. The source of triglyceride (Calbiochem triglyceride) was at 43.39 mg/mL, which was used at 40ul/mL DMEM as high triglyceride, corresponding to 173 mg/dL. After experimental medium

addition, wells were observed every day for nodule formation. Cells from each experimental condition and control were evaluated for calcium deposition, at day 3 after initial nodule formation by the control cells. 5 mM β-glycerophosphate was added to wells at 3 days prior to calcium assays, to allow a measurable level of calcium. Control wells without added 5 mM β-glycerophosphate were also evaluated for both nodule counts and calcium deposition, to control for any influence this substance had in the presence of experimental factors. Statistical analysis revealed whether the number of nodules formed was directly correlated to the amount of calcium deposition, which would have established the counting of nodules as an endpoint for easily evaluating calcium deposition. In subsequent experiments at both high and low glucose concentrations, the effect of factors on the average number of nodules formed per well, the rate of nodule formation, calcium deposition and the presence of calcium in and around nodules were determined.

2.5 Nodule Visualization

Nodules were observed and counted in wells using an Olympus light microscope at overall magnification of 40X and 100X.

2.6 Histology

Cells were stained by standard methods for smooth muscle cell phenotype indicators including anti-smooth muscle α-actin (positive for SMCs) and von Willebrand stain (v WF or Factor VIII complex, negative for SMCs). Anti-smooth muscle α-actin (clone 1A4) and polyclonal anti-human von Willebrand factor were acquired from Sigma (St. Louis, MO).

2.61 α-actin and vWF Stains

To identify SMCs, monolayers were stained with anti-smooth muscle cell α-actin (clone 1A4, 2.5 *u*L/mL PBS) or von Willebrand Factor (1.33 *u*L/mL PBS). After a wash with

PBS-T, coverslips were incubated for 30 min with protein A-horseradish peroxidase, and then washed again. Staining was visualized using horseradish peroxidase substrate DAB (Sigma Fast DAB, Sigma, St. Louis, MO). The development was stopped with water and slides were counterstained with nuclear fast red. Controls were done by replacing either the antibody or protein A-HP with PBS-T followed by DAB development.

2.62 Qualitative Calcification Visualization/Von Kossa Stain

Cell monolayers on tissue culture treated coverslips were fixed with Histochoice (Amresco) followed by Von Kossa staining to assess the presence or absence of calcium in and around SMC nodules. Slides were flooded with 5% aqueous silver nitrate solution, followed by exposure to UV light for 10-20 min. Slides were then washed 3 times with deionized water. Unreacted silver was removed by incubating slide for 2 min with 5% aqueous sodium thiosulfate, followed by another three washes with deionized water. Counterstaining was done using nuclear fast red exposure for 3-5 min. This technique can be found in *Theory and Practice of Histotechnology*, 2nd edition, by Sheehan and Hrapchak, copyright 1980 by Battelle Press.

2.7 MTT Assay

The MTT assay was used to verify non-toxic conditions by measuring mitochondrial metabolic activity. MTT is a dye that is oxidized by the mitochondria of living cells. 100 uL of SMCs at 2 x 10^5 cells/mL were seeded in 96-well flat-bottom microtiter plates for 24 hr at 37°C prior to lipid application for 48hr. 25 *u*L of 5 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) in deionized water was then added to all wells except blanks for 2 hr at 37°C. 100 *u*L of extraction buffer [20% sodium dodecylsulfate (SDS)/50% N, N-dimethyl-formamide (DMF)] was then added to all wells for 12 hr (overnight). Blue wells indicated living cells, yellow wells contained dead cells. The microtiter plate was then read at 590 nm on a UNISKAN II microplate reader (McLean, VA). Control wells contained cells with normal medium, without additional lipid. Other controls included wells with no cells with no MTT added, wells

with no cells plus MTT, and wells with cells killed with 50% ethanol plus MTT (dead cells).

2.8 Calcium Quantification Assay

Cells in 24-well plates treated as described in section **2.4** were seeded and fed with growth medium supplemented with 5mM β-glycerophosphate 3 days prior to the calcium assay. After seeding for 24 hr, lipid in calcification medium was added to experimental wells. 48-72 hours after addition of lipid, wells showed nodules in approximately the same distribution as reported for nodule formation counts, with high additional cholesterol wells showing the greatest number of nodules. Cells were incubated with 0.6 N HCl for 24 hr to solubilize calcium. The digested samples were assayed for calcium using the o-cresolphthalein complexone reaction which complexes with calcium in alkaline medium to produce a colorimetric change at 575 nm that was read using a Beckman DU-6 spectrophotometer with a 570 nm filter.

3.0 Statistical Analysis

Effects analyzed statistically were: a.) nodule counts between differently treated samples, b.) calcium deposition (calcium assay) between differently treated samples, c.) metabolic activity (MTT assay) between differently treated samples. All experiments were performed in at least triplicate. MTT and calcium assays were also done in duplicate plates, which demonstrated reproducibility of these assays. Statistical variance (P) was determined by the t-test: two sample assuming unequal variances, with statistically significant results defined as $p<0.05$.

4.0 Results

4.1 Establishment of the Experimental Model

SMCs formed complex multicellular nodules in post-confluent cultures that stained poorly for α-actin and accumulated calcium deposits.

It was previously established that sheep aortic SMCs are α-smooth muscle cell actin positive and von Willebrand factor negative, and at high passage number (8 to 13) are capable of forming multicelluar nodules that calcify. Sheep aortic fibroblasts and endothelial cells were incapable of nodule formation, suggesting that nodule formation only occurs in certain cell types and is not an artifact of tissue culture technique. SMCs were grown in medium past confluence to develop nodules. Nodules were multi-cellular aggregations of cells. In this study, the nodules were then stained for indicators of SMC characteristics and with Von Kossa to visualize nodule calcification.

Initial experiments were conducted to establish the appropriate growth medium and conditions for nodule formation. Because the level of fetal bovine serum (FBS) differs in several *in vitro* nodule formation models, cells seeded at $2x10^5$cells/mL were exposed to FBS at three different levels, 10%, 15%, 20%, in growth medium, to determine if FBS affected the number and/or rate of nodule formation. A level of 10% FBS resulted in the greatest number and fastest formation of nodules and was therefore used as the level of FBS in growth media.

Experiments initially using RPMI medium, chosen to allow for varying glucose levels, revealed that RPMI did not support nodule formation at glucose levels from 0 to

550 mg/dL (data not shown). DMEM at both low (100 mg/dL) and high (450 mg/dL) glucose was then selected.

Initially, 153 mg/dL and 18.84 mg/dL were chosen as the high and low cholesterol levels. However, this level of high cholesterol (153 mg/dL) would not support a SMC monolayer and appeared toxic to cells. Therefore 48.72 mg/dL was used as the high concentration for cholesterol. Low cholesterol was kept at 18.84 mg/dL cholesterol. High cholesterol was three times the amount (45uL) of the cholesterol-rich lipid source as low cholesterol (15uL) in one mL DMEM.

Nodules were seen in SMC cultures grown in DMEM at approximately 7 days post-confluence. SMC cultures stained positive for α-actin (Figure 1.B.) and negative for von Willebrand Factor (Figure 1.A.), an endothelial cell marker, thus establishing these cells as SMCs. Nodules did not stain well with α-actin (Figure 1.C.), indicating a possible phenotypic change. The nodules tested positive for calcium using the Von Kossa stain (Figure 1.D.). SMCs had to be grown in calcifying medium, containing 5mM beta-glycerophosphate, for at least 3 days prior to staining to obtain a test positive for calcium by the Von Kossa stain. Those cells capable of nodule formation were chosen for subsequent experiments. The center, but not the periphery, of nodules stained positive using trypan blue exclusion, whereby dead cells retain a blue color (Figure 1.E.).

4.2 High Glucose Results

Cholesterol increased nodule formation in a dose-dependent manner.

Figure 2.A. shows that addition of cholesterol at both low (18.84 mg/dL) and high (48.72 mg/dL) levels led to an increase, approximately 7-fold and 21-fold respectively, in nodule formation over control (3.9 mg/dL, $p<0.05$). The control value was 22.3 nodules formed per well at high glucose without additional lipid. The significant three-fold difference between the cholesterol effects on nodule formation corresponds with the three-fold differences in additional cholesterol, suggesting a dose-dependent relationship between cholesterol and nodule formation.

Triglyceride increased nodule formation, but not to the magnitude of the cholesterol effect.

Figure 2.A. demonstrates that high triglyceride (173 mg/dL) treatment significantly increased nodule formation approximately three-fold over control. Both high and low cholesterol led to significantly more nodule formation than high triglyceride.

Cholesterol increased the rate of nodule formation, in addition to the number of nodules formed.

Increased nodule formation by high cholesterol over control was observed 24 hr before any significant increase in nodule formation over control by either low cholesterol or high triglyceride (Figure 2.B.). Triglyceride only increased nodule formation at 48 hr after lipid addition.

Cholesterol exhibited no significant effect on SMC calcification at high glucose.

Cholesterol added to SMC cultures grown in calcification media (DMEM plus 5mM b-glycerophosphate) at elevated levels of glucose (450 mg/dL) showed that there were no significant differences in calcium deposition from the control value with either low or high cholesterol treatment, as shown in Figure 3.

Cholesterol increased metabolic activity more than triglyceride at high glucose.

Metabolic activity was measured by an MTT assay to determine if lipid levels were toxic to cells. As shown in Figure 4, metabolic activity increased 1.33-1.35 times with either low or high cholesterol treatment over the control value ($p<0.05$). However, there was no significant difference (<2%) in metabolic activity between low and high cholesterol treatments. Metabolic activity significantly increased 1.21 times over control with the addition of high triglyceride (Figure 4). This increase in metabolic activity with high triglyceride treatment was to a small (10-12%), but significant, magnitude less than with either low or high cholesterol treatment ($p<0.05$). The levels of lipids applied were not toxic to the cells at high glucose.

4.3 Low Glucose Results

High cholesterol increased nodule formation much more than either low cholesterol or triglyceride at low glucose.

In order to determine the influence on nodule formation at low levels of glucose (100 mg/dL) exerted by certain factors associated with increased dystrophic calcification, cholesterol and triglyceride were added as described previously, this time to SMC cultures seeded for 24 hours in high glucose DMEM, then fed with or without lipid in low glucose DMEM.

At low glucose, the 2.4-fold increase in nodule formation over the control (8.7 nodules) upon treatment with low cholesterol was not significant (Figure 5.A.). However, nodule formation increased 28-fold over control with the addition of high cholesterol ($p<0.05$). There were 12 times more nodules with high cholesterol treatment than with low cholesterol treatment ($p<0.05$).

At low glucose, there were no significant differences in the number of nodules formed between the following treatments (Figure 5.A.): control and high triglyceride, low cholesterol and high triglyceride and between control and low cholesterol. At low glucose, as previously seen at high glucose (see Figure 2.A. for this 7-fold increase), high cholesterol increased nodule formation significantly (22.4 times) more than high triglyceride.

High cholesterol increased nodule formation to a greater magnitude in low glucose than in high glucose conditions.

As shown in Figure 5.B., there were 18.2 times more nodules upon treatment with high cholesterol than the control value of 5.3 nodules. In comparing low glucose with high glucose results in terms of nodule formation, Figure 5.B. shows that there were no significant differences between low and high glucose at control, low cholesterol and high triglyceride. The most striking result, as demonstrated in Figure 5.B., was at high cholesterol, there were 2.5 times more nodules formed at low glucose than at high glucose ($p<0.05$).

There were no significant differences from control in the number of nodules formed with high triglyceride treatment at either low or high glucose (Figure 5.B.). However, Figure 2 showed a slight (3-fold) significant increase in nodule formation at high glucose with high triglyceride over control. At low glucose, there was no significant change (1.9-fold) between low cholesterol and high triglyceride treatments, unlike at high glucose where 3.2-fold and 2.3-fold increases with low cholesterol were seen over high triglyceride in Figure 5.B and Figure 2, respectively. At low glucose, there was a significant (22.4-fold) increase in nodule formation with high cholesterol versus high triglyceride treatment, an increase also seen at high glucose in both Figure 5.B. (11.2 times) and in Figure 2.A. (7 times). At low glucose, cholesterol increased the rate of nodule formation compared to control within 24 hr. Within 48hr, this observation was also made at high glucose (Figure 5 C).

Cholesterol did not cause significant changes in calcium deposition by SMCs under low glucose conditions.

In order to determine the influence on calcium deposition and metabolic activity at low glucose (100 mg/dL) exerted by certain factors associated with increased dystrophic calcification, cholesterol and triglyceride were added to SMC cultures seeded 24 hr in high glucose DMEM, then fed with lipid in low glucose DMEM. Calcium deposition was then measured after dissolving the monolayer in 0.6N HCl for 24 hr.

The addition of cholesterol did not result in any increase in calcium deposition at low glucose (Figure 6.A.). There were no significant changes in calcium deposition between control and low or high cholesterol treatments, or between low and high cholesterol treatments.

Figure 6.B. shows the results of calcium assays with lipid treatments at both low and high glucose. There was no significant difference between low glucose and high glucose controls. Glucose alone or cholesterol alone did not influence calcium deposition (Figure 6.B.). At high glucose, either low or high cholesterol seemed to increase calcium deposition. At low glucose, the presence of either low or high cholesterol appeared to decrease calcium deposition. Decreased glucose in the presence of either low or high cholesterol exhibited an inverse relationship to calcium deposition. With additional cholesterol, lowering glucose appeared to decrease calcium deposition by 2.6-2.7 fold ($p>0.05$). However, the control values (absorbance at 570 nm) under low glucose and high glucose showed large variation, so the glucose effect on calcium deposition was inconclusive.

Cholesterol increased metabolic activity at low glucose, whereas triglyceride did not increase metabolic activity.

Figure 7.A. shows the effect of lipid on metabolic activity under conditions of low glucose. At low glucose, there was a slight (0.95 times) decrease from control upon high triglyceride treatment. There was a 1.42-fold increase in metabolic activity upon high cholesterol treatment over control and a 1.49-fold increase with high cholesterol versus high triglyceride treatment ($p<0.05$). The levels of lipids applied were not toxic to the cells at low glucose.

High glucose alone increased metabolic activity.

The lipid effect on metabolic activities at both low and high glucose is shown in Figure 7.B. Control values (absorbance at 590 nm) were 0.750 for low glucose and 0.805 for high glucose. High glucose control was 1.07 times greater than low glucose control ($p<0.05$), thus high glucose alone increases metabolic activity. At high glucose, significant 1.33 and 1.2 times increases in metabolic activity over control were observed with high cholesterol treatment and with high triglyceride treatment, respectively. Treatment with high cholesterol gave 1.1 times (10%) higher metabolic activity than treatment with high triglyceride ($p<0.05$). A significant 1.36-fold decrease in metabolic activity from high glucose to low glucose conditions with high triglyceride was noticeable. There was no significant difference between low and high glucose in metabolic activity with high cholesterol treatments.

5.0 Figures

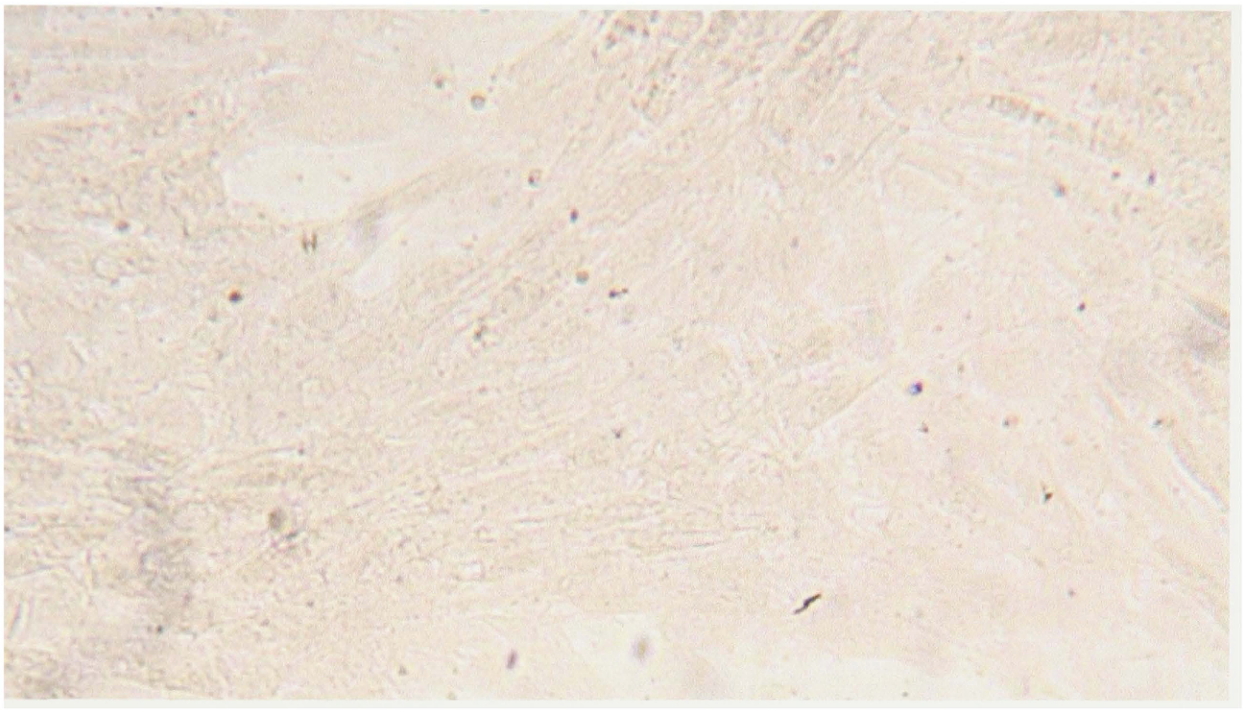

Figure 1.A. Sheep smooth muscles cells did not stain positive for von Willebrand factor 7 days after confluence. Magnification 40X.

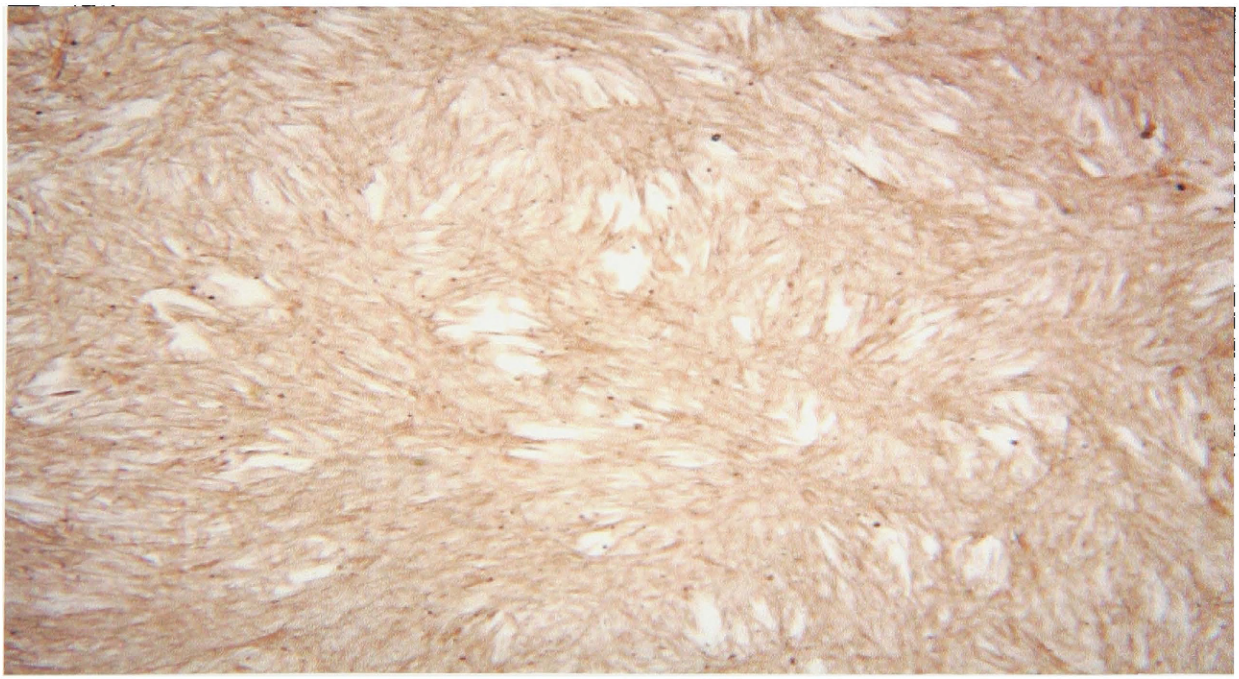

Figure 1.B. Sheep smooth muscles cells stained positive for smooth muscle cell α-actin 7 days after confluence. Magnification 40X.

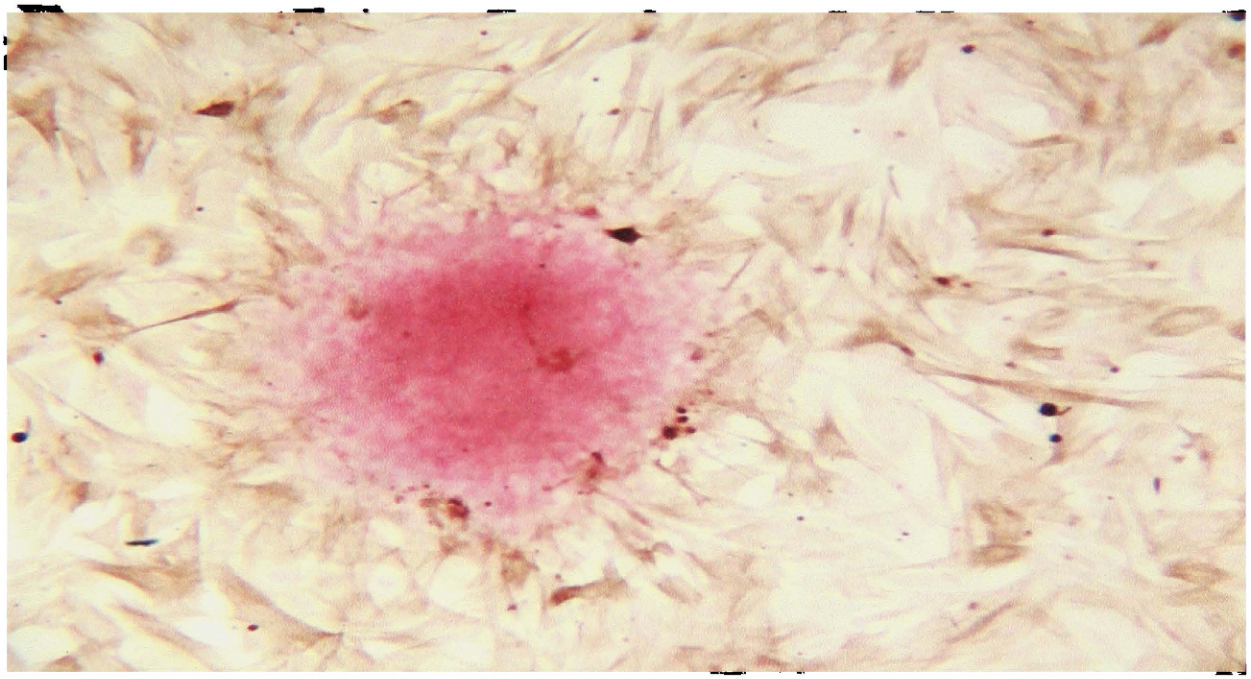

Figure 1.C. Sheep smooth muscles cells and nodule stained for smooth muscle α-actin, counterstained with nuclear fast red 7 days after confluence. The nodule did not stain positive for α-actin, whereas the surrounding SMCs did. Magnification 40X.

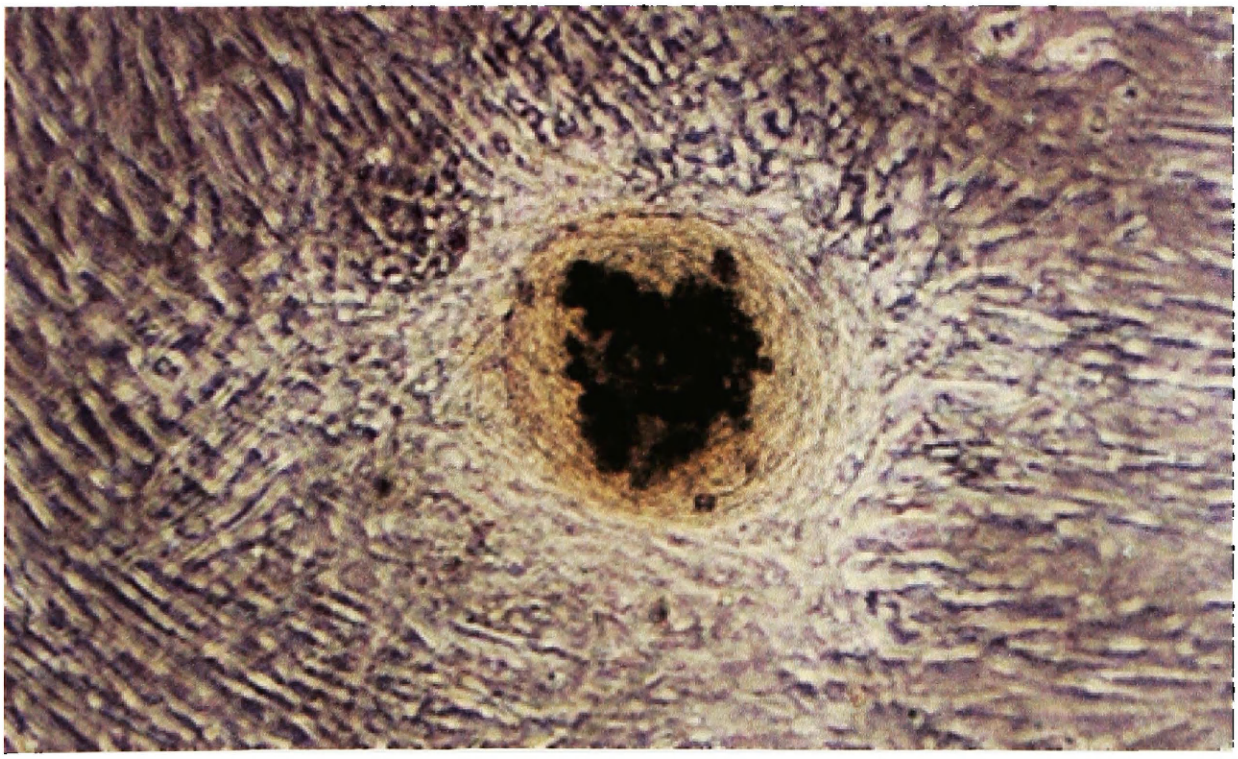

Figure 1.D. Sheep smooth muscles cells and nodule stained by Von Kossa 7 days after confluence, 3 days after application of calcification media. Brown areas, concentrated within the nodule, are calcified. Magnification 40X.

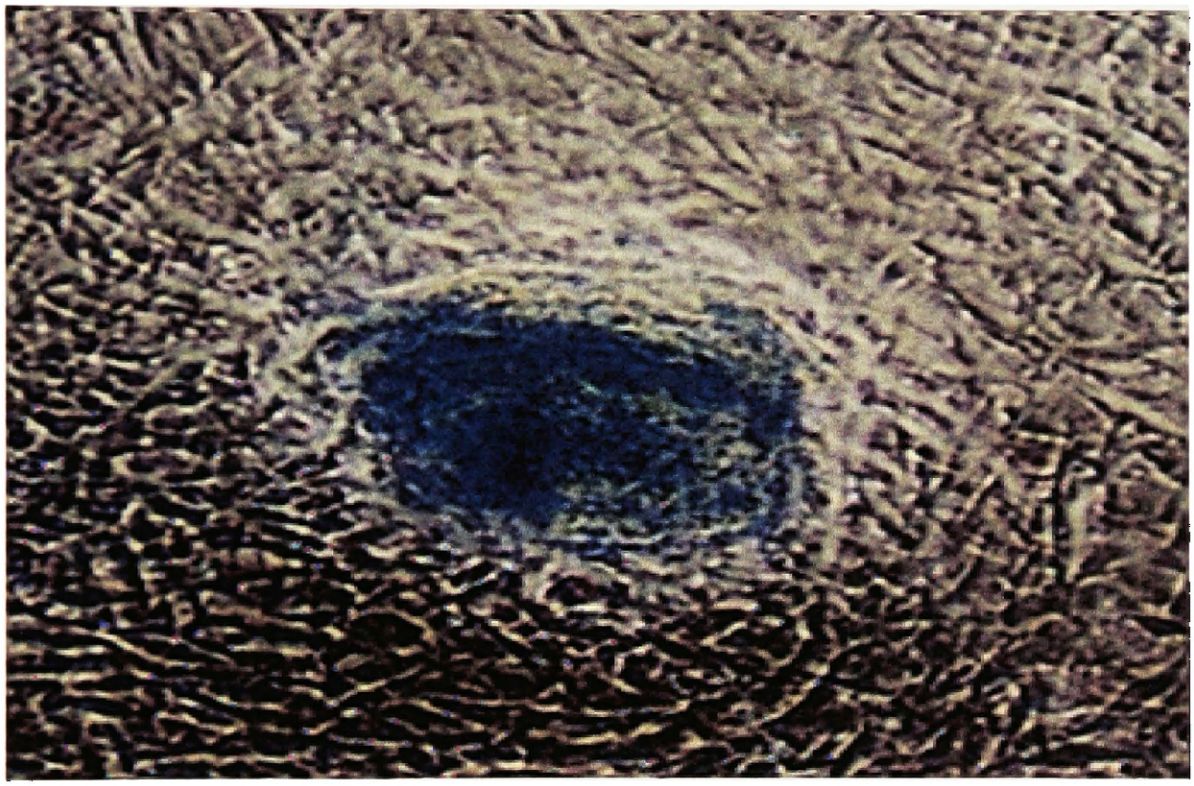

Figure 1.E. Sheep smooth muscles cells and nodule stained by trypan blue exclusion 7 days after confluence. Blue cells, concentrated in the nodule, are non-viable. Magnification 40X.

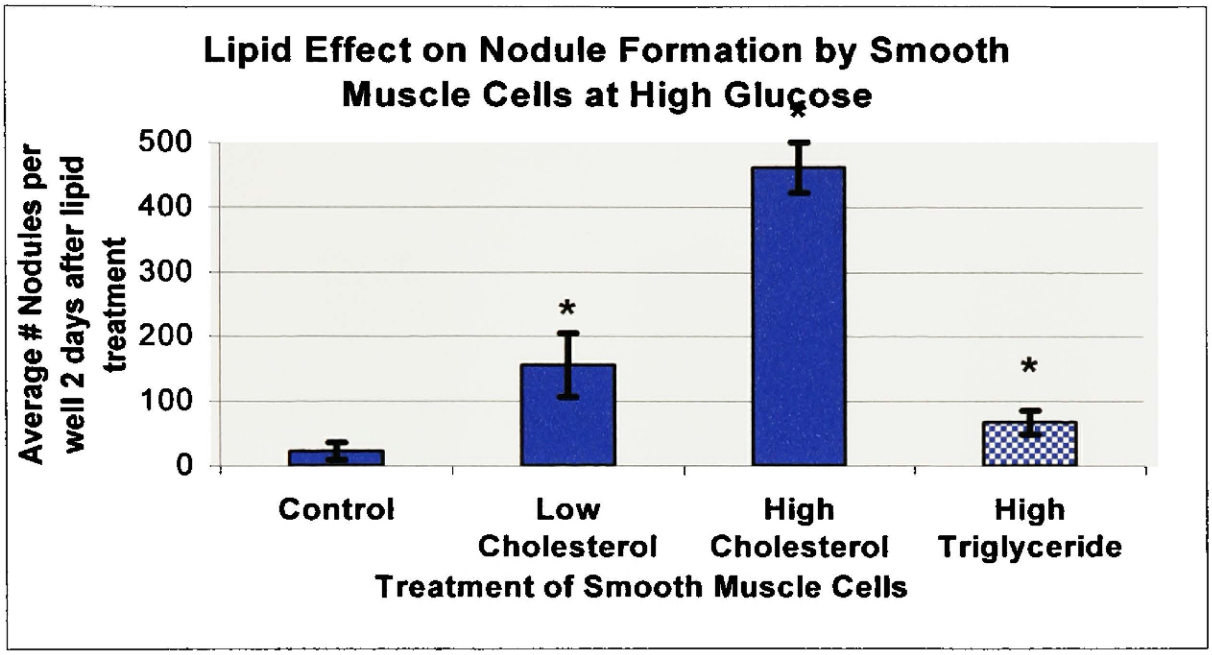

Figure 2.A. Lipid effect on the average number of nodules formed per well by SMCs at high (450 mg/dL) glucose, two days after lipid treatment. Error = s.d., * = significant increase over control, n = 6.

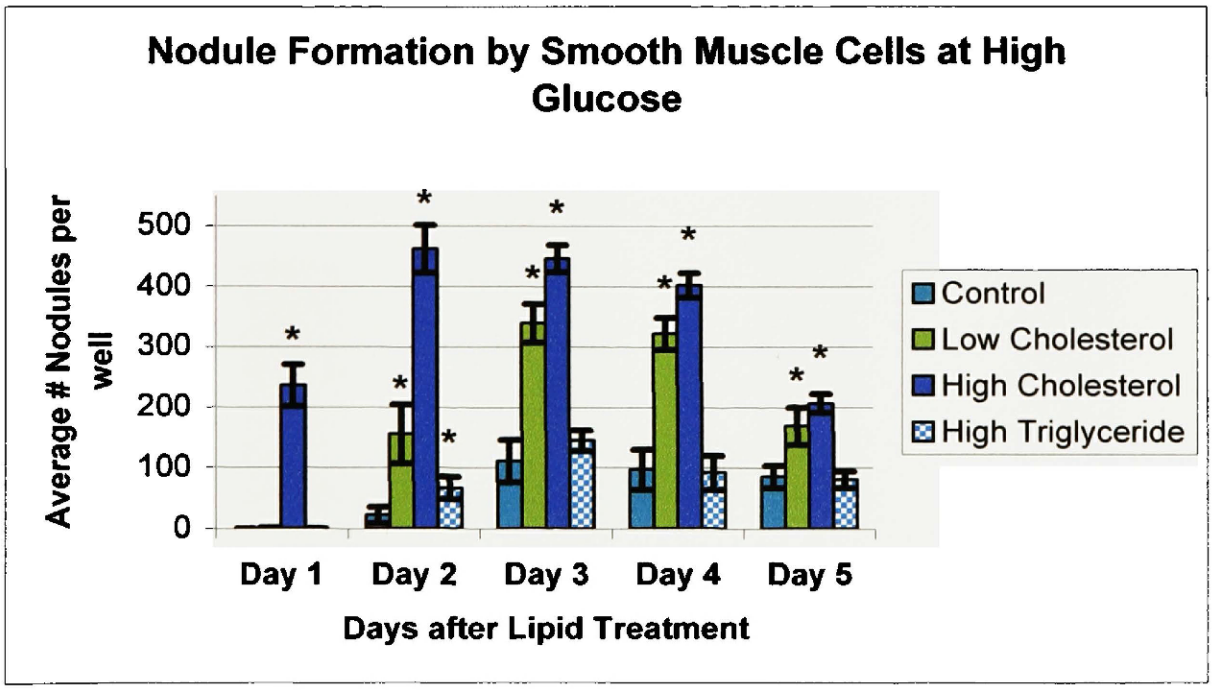

Figure 2.B. Lipid effect on the average number of nodules formed per well by SMCs at high glucose, one to five days after lipid treatment. Error = s.d., * = significant increase over control, n = 6.

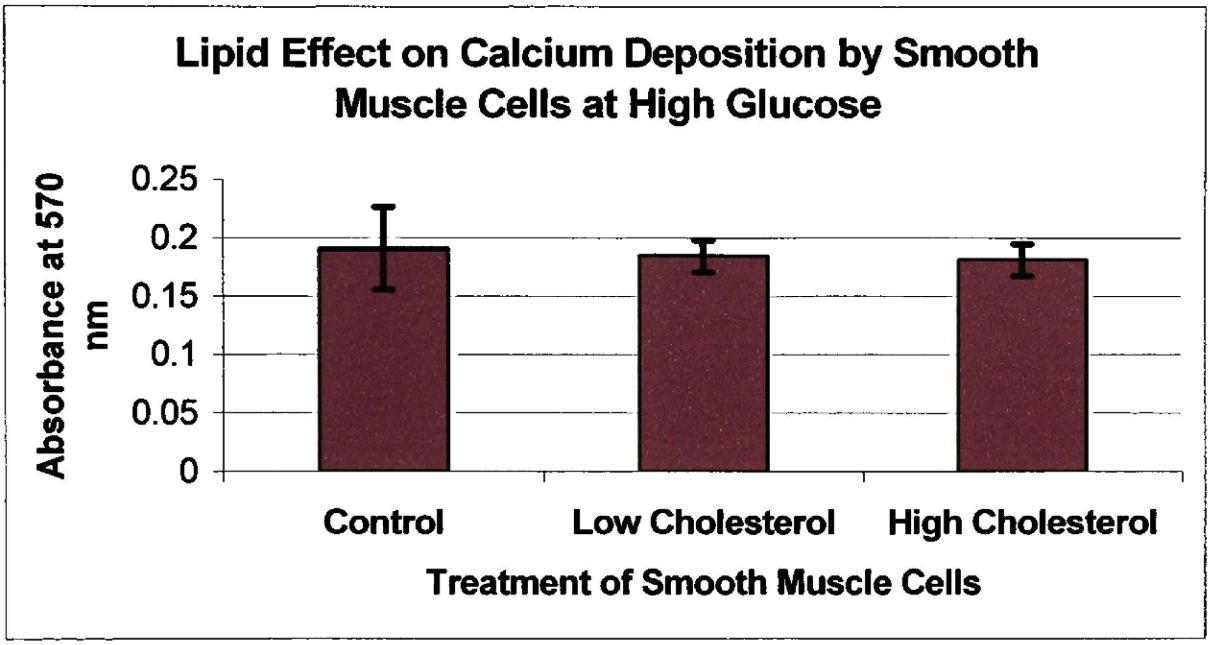

Figure 3. Calcium deposition by SMCs 2 days after treatment with lipid at high glucose, as measured by the average absorbance at 570 nm per well. Error = s.d., n=8, p>0.24.

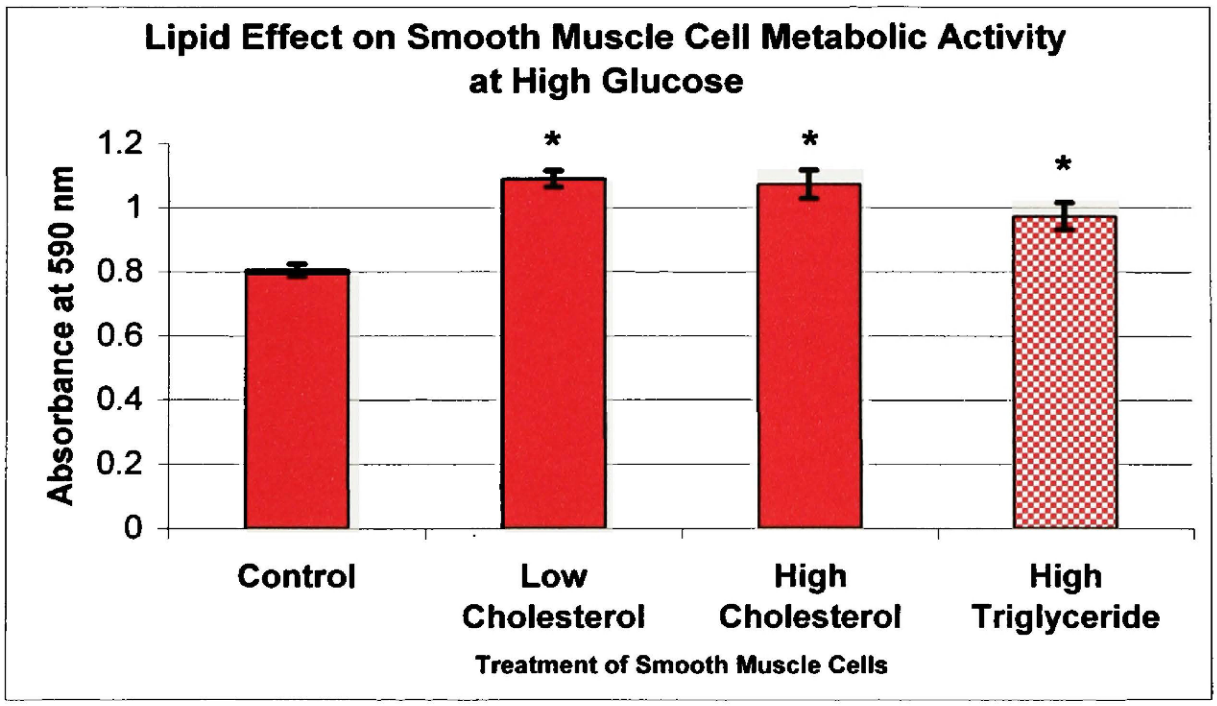

Figure 4. Lipid effect on metabolic activity at high glucose, as measured with MTT assay as the average absorbance at 590 nm per well. Error = s.d., n=8. *=p<0.005 over control.

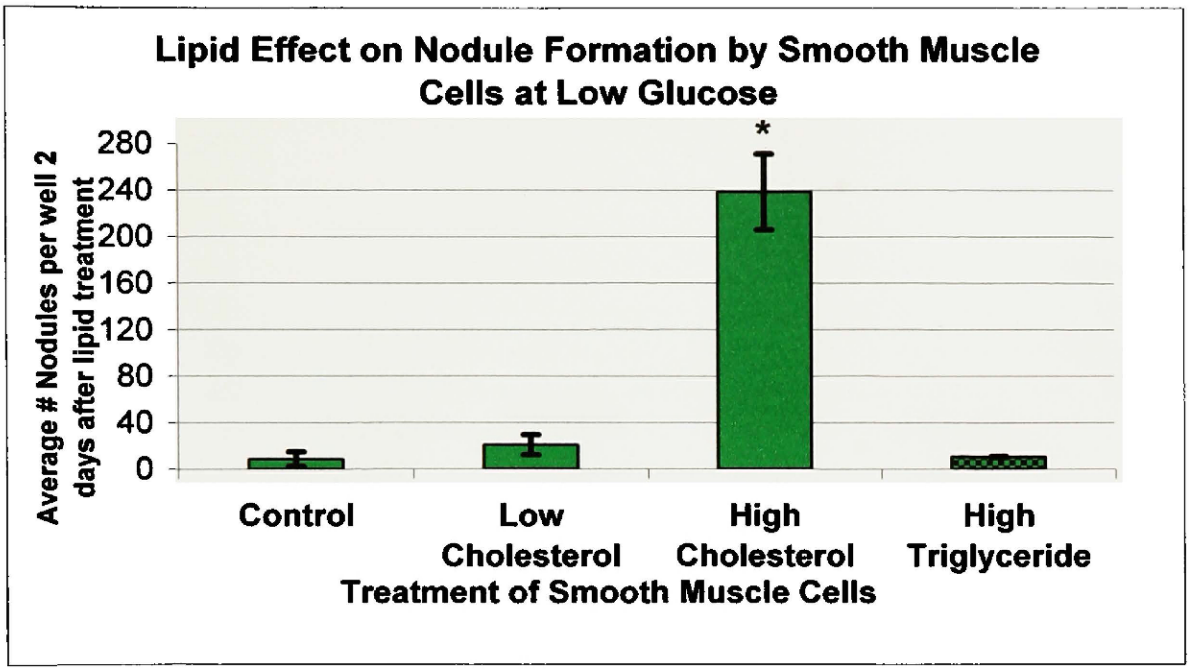

Figure 5.A. Lipid effect on the average number of nodules formed per well by SMCs at low glucose 2 days after lipid treatment. Error = s.d., * = significant increase over control ($p<0.05$), n = 3.

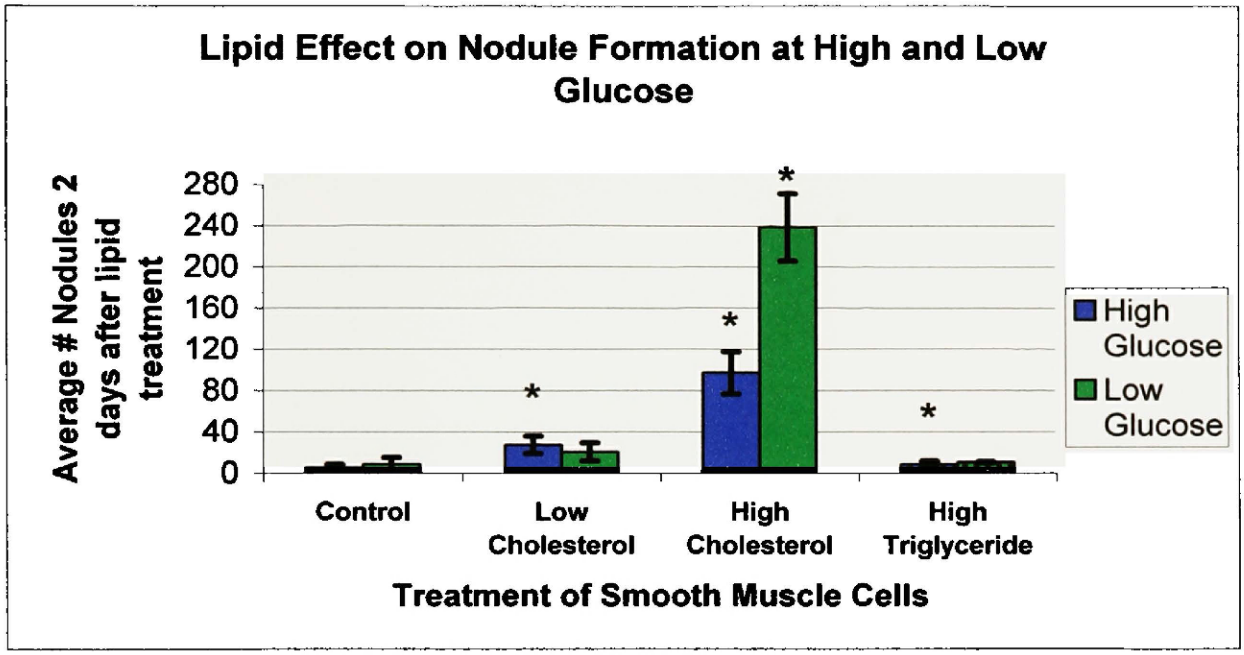

Figure 5.B. Lipid effect on the average number of nodules formed per well by SMCs at low and high glucose 2 days after lipid treatment. Error = s.d., * = significant increase over control ($p<0.05$), n = 3.

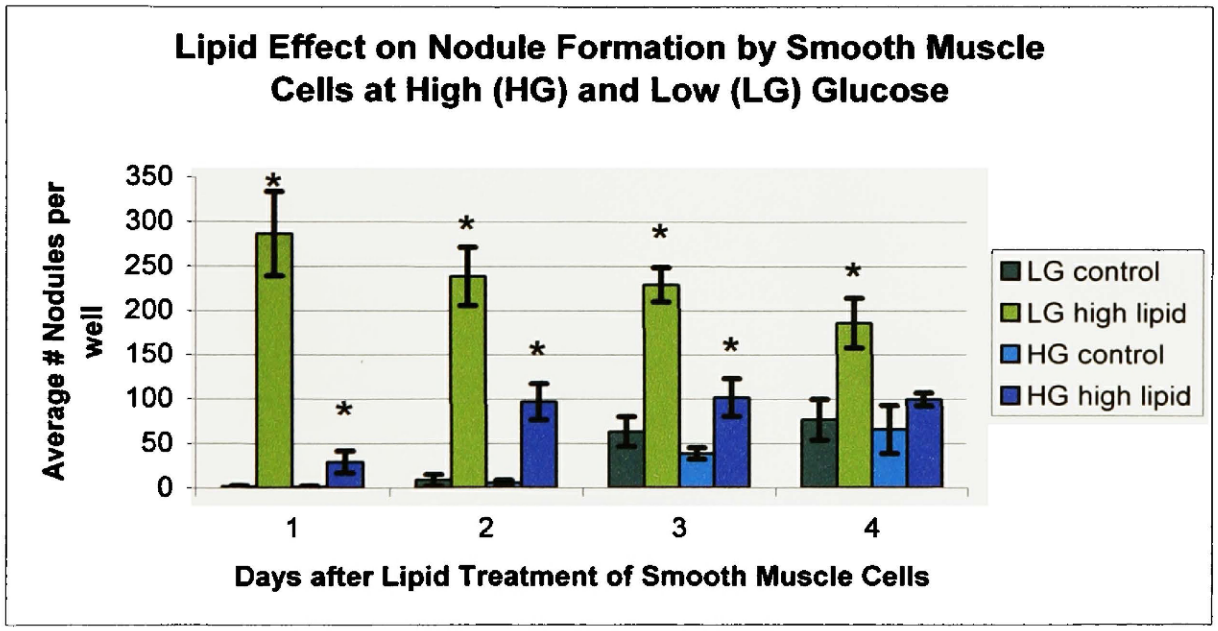

Figure 5.C. Lipid effect on the average number of nodules formation by SMCs at low and high glucose from one to four days after lipid treatment. Error = s.d., * = significant increase over control ($p<0.05$), n = 3.

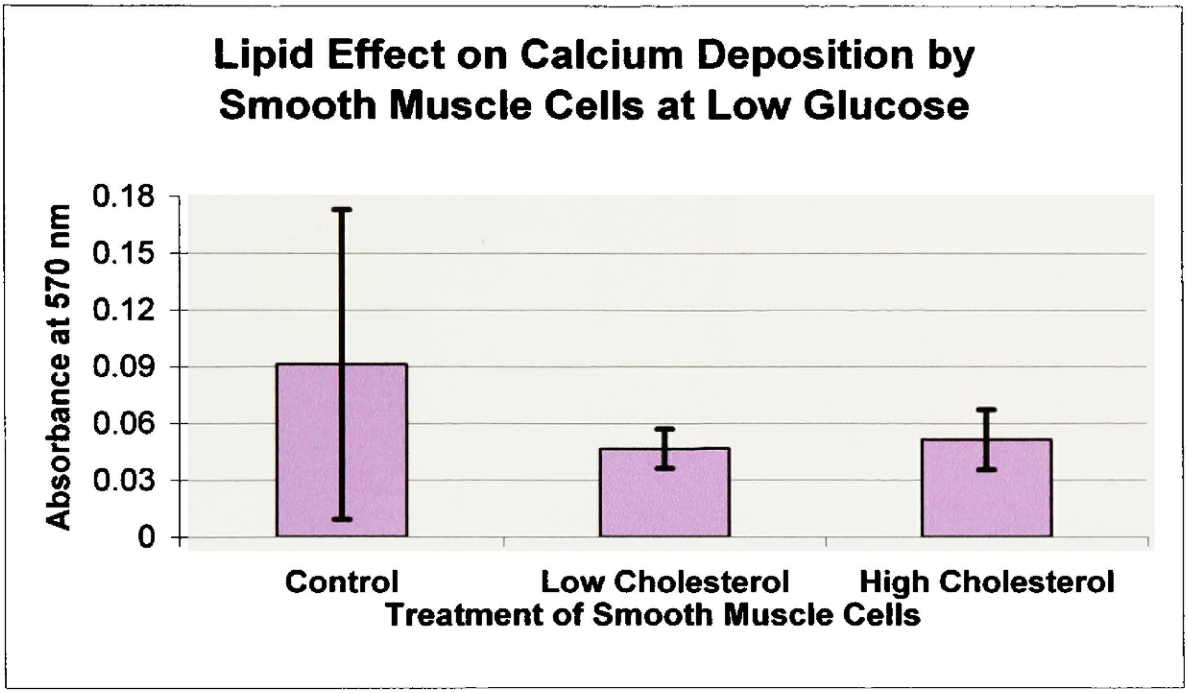

Figure 6.A. Calcium deposition by SMCs 2 days after treatment with lipid at high glucose. Error = s.d., n=8, p>0.08.

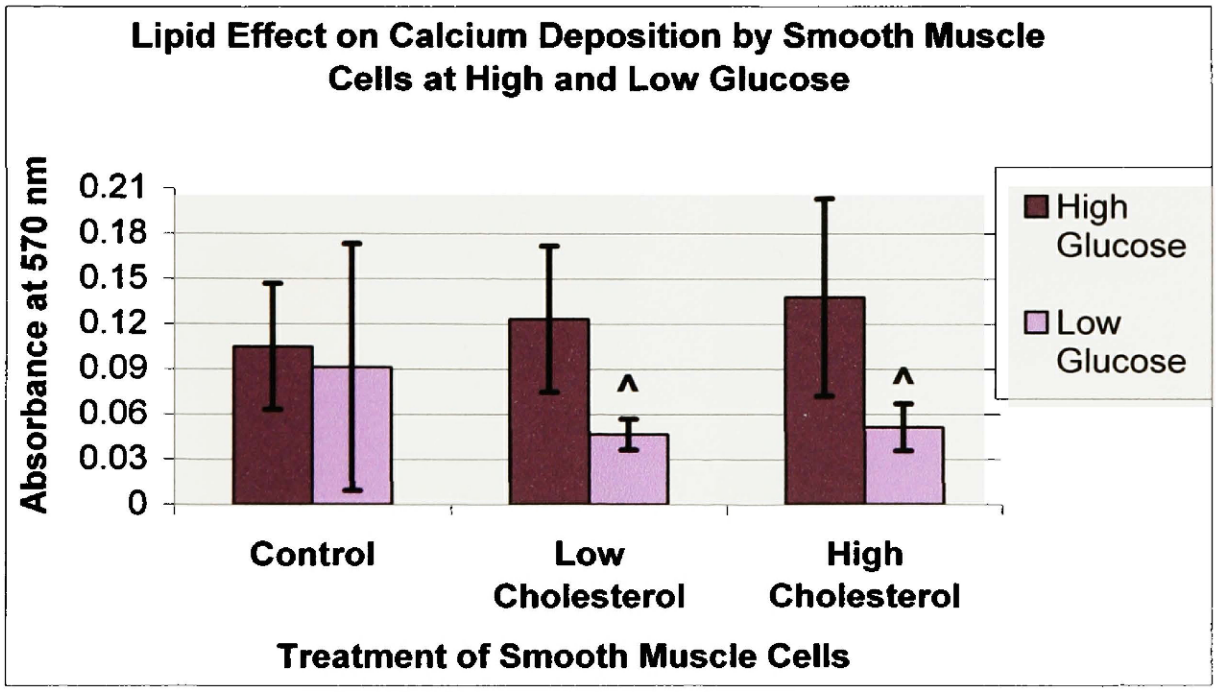

Figure 6.B. Calcium deposition, as measured by absorbance at 570 nm, by SMCs 2 days after treatment with lipid at low and high glucose. Error=s.d., n=8, ^ denotes significant decrease at low versus high glucose.

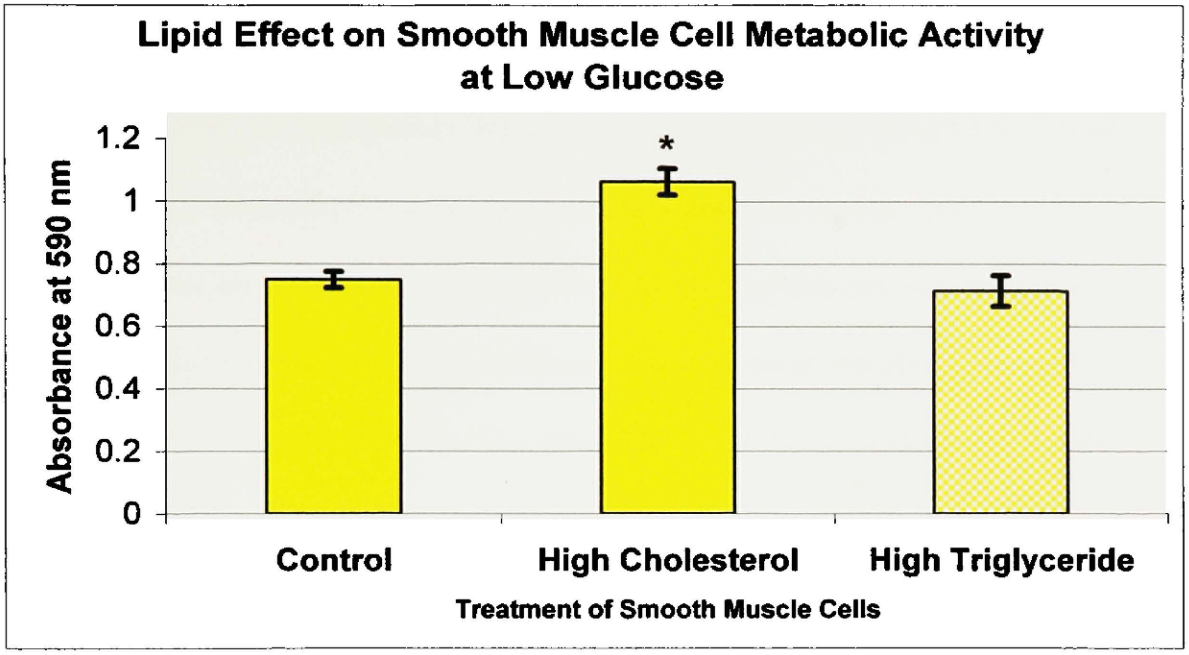

Figure 7.A. Metabolic activity of SMCs, two days after lipid treatment, as measured with MTT assay. Error=s.d., n=8, *=p<0.046 over control.

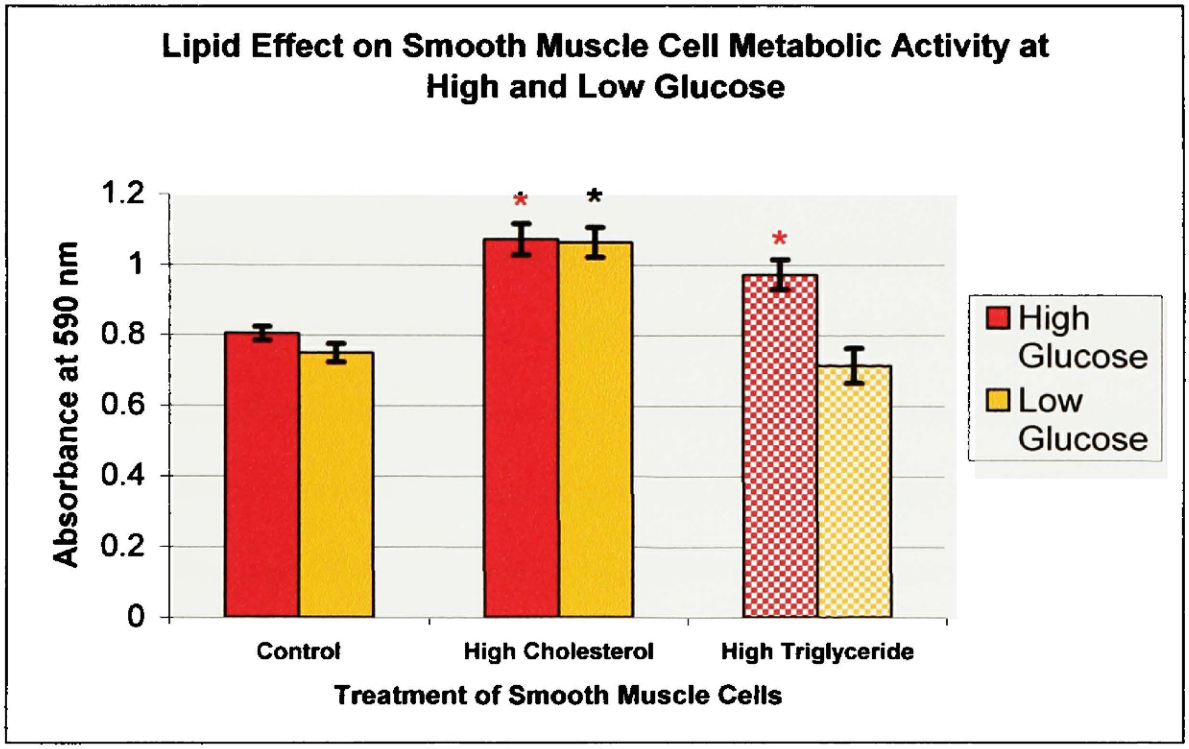

Figure 7.B. Metabolic activity of SMCs at high and low glucose, as measured with MTT assay as absorbance at 590 nm, two days after lipid treatment. Error = s.d., n=8. * = significant increase over control.

6.0 Discussion

The present investigation was aimed at understanding some of the cellular mechanisms of atherosclerotic plaque formation and subsequent calcification, in particular those serum factors that may mediate these processes that are present at elevated levels as seen in diabetes mellitus, a disease state closely associated with increased rates of atherosclerosis. The hypothesis was that serum factors found at elevated levels during diabetes mellitus, such as lipid and glucose, would increase both nodule formation and calcification, thereby suggesting a role in atherosclerosis *in vivo*.

Cholesterol increased the number of nodules formed, but did not increase or influence calcification of these nodules at either low or high glucose levels. The increase in nodule formation found at high cholesterol levels was influenced by glucose concentration, with low glucose causing a greater number of formed nodules than at high glucose.

The hypothesis that increased lipid leads to increased nodule formation was supported. At high glucose, addition of cholesterol or triglyceride increased nodule formation. Cholesterol resulted in a more significant increase in nodule formation than triglyceride. Nodule formation appeared to have a dose-dependant positive response to cholesterol. Thus, specific lipids at particular levels may influence an increase in atherosclerotic lesion formation *in vivo*.

The Demer group has demonstrated that CVCs increased nodule formation upon application of an oxidized form of cholesterol, 25-hydroxycholesterol (25-OHC) [13]. From the results presented in this thesis, it appears that unoxidized cholesterol is also capable of increasing nodule formation. However, it is possible that cholesterol in our

system was converted to the oxidized form. An experiment that tested the effect of both unoxidized and oxidized cholesterol on nodule formation using this system would indicate whether further tests on oxidation state were required. The hypothesis would be that oxidized cholesterol causes an increase in calcification and unoxidized cholesterol causes no change in calcification. If both oxidized and unoxidized cholesterol both changed or did not change calcification, then further testing of the oxidized states of the cholesterol would be required. Other lipids have been shown to increase both nodule formation and calcification by vascular cells, particularly 25-hydroxycholesterol (25-OHC) [13]. Many researchers have explored the effect of oxidized lipids on vascular cells. Other oxidized lipids, such as ox-LDL, were shown to increase nodule formation by vascular cells [5]. In the experiments with ox-LDL, researchers found no increase in nodule formation by unoxidized lipids [5]. Our research demonstrates that at either high or low glucose, the unoxidized lipid cholesterol increased the number of nodules formed, whereas triglyceride lipids had little or no influence on nodule formation.

The addition of low or high cholesterol or triglyceride showed no significant changes in nodule calcium deposition over control. Cholesterol or lipid did not influence calcification at either low or high glucose, which contradicts our hypotheses regarding calcification. Contrary to our hypothesis, nodule count and calcium levels found in culture do not appear to be related. This differs from previous findings, where serum factors, such as 25-OHC, were found to increase both nodule formation and calcium deposition by CVCs[13]. *In vitro*, oxidized forms of cholesterol can increase both nodule formation and calcification, whereas unoxidized cholesterol only appears to increase nodule formation with no change in calcification. *In vivo*, increased cholesterol in serum

contributes to early abnormalities in VASMCs that develop into atherosclerotic lesions. Over time, cholesterol accumulating in these lesions becomes oxidized, and perhaps it is the oxidized form of cholesterol, 25-OHC, rather than the unoxidized form, which contributes to increased calcification of the atherosclerotic plaque. Data collected from *in vivo* atherosclerotic plaques revealed increased levels of 25-OHC. This, along with the current data, suggests that *in vivo*, unoxidized cholesterol can increase atherosclerotic lesion formation, but that the oxidized form of cholesterol, formed later, is required to increase calcification of atherosclerotic lesions and plaques.

Oxidized lipids were chosen by previous groups due to their known role in osteoblast stimulation during bone formation. This connection to osteoblasts was made by other groups, including Demer et al, when smooth muscle and other vascular cells were shown to undergo a phenotypic change in the direction of osteoblasts when forming nodules [6, 7, 12,13]. Our data indicated a possible phenotypic change in cells involved in nodule formation, as nodules did not stain well with α-actin. Other phenotypic markers such as alkaline phosphatase (ALP) would provide a better indication whether phenotypic change was in the direction of osteoblasts. Other groups, including Demer et al, noted this phenotypic change in the course of nodule formation and suggested differentiation of SMCs toward osteoblast-like cells [12,13]. The differentiated state of intimal SMCs within atherosclerotic lesions *in vivo* is often altered. Several groups have noted osteoblast-like characteristics developed in those cells forming nodules, including loss of normal SMC markers, and increased expression of osteoblast markers such as ALP. Anti-H-caldesmon may also be used to verify SMC phenotype and to distinguish from myofibroblasts and pericytes.

Previous research on the lipid effect on nodule formation and calcification by vascular cells was nearly exclusively carried out *in vitro* at diabetic or higher levels of glucose. To ascertain whether the increased nodule formation at high cholesterol was influenced by glucose concentration, nodules subjected to high cholesterol and control (no additional lipid) were counted at both low (100mg/dL) and high (450mg/dL) levels of glucose. There was an influence of glucose on nodule formation, yet this influence was in conjunction with high cholesterol. At high cholesterol, the number of nodules formed at low glucose was considerably more than the number formed at high glucose. Cholesterol at low glucose increased nodule formation only when at a level which was nearly 3 times higher than that required for significant increase on nodule formation seen at high glucose. This suggests that high glucose stimulates the increased effect of low levels of cholesterol on nodule formation, i.e. glucose and low cholesterol appear to work in a cooperative manner on nodule formation. Nodule formations at both high and low glucose (see Figures 2 and 5.B.) were significantly increased over controls with the addition of high cholesterol, and there was a significant increase in nodule formation between low and high cholesterol. However, under conditions of high cholesterol *in vitro*, decreased levels of glucose (100mg/dL) demonstrated a greater increase in nodule formation over controls than observed at elevated glucose (450mg/dL). Previous studies have been conducted at high glucose levels and have failed to explore this cholesterol-glucose relationship.

The cholesterol effect on nodule formation by ASMCs was clearly influenced by the concentration of glucose. This may be related to situations seen *in vivo* during prolonged diseased states. In diabetes mellitus, prolonged elevated serum levels of

glucose affect insulin levels and/or functionality, which creates a starvation state for vascular cells, i.e. glucose is unable to be brought into cells with the help of insulin at normal (non-diseased) rates. Perhaps this *in vitro* increase in the high cholesterol effect on nodule formation at low glucose versus high glucose reflects an increased effect of cholesterol on SMC abnormalities (atherosclerotic lesions) when adequate glucose is not available to vascular cells. There is an underlying implication that alternate metabolic pathways, i.e. those utilizing cholesterol versus glucose, may contribute to nodule formation, or are at least somehow involved, perhaps through glycolytic metabolite shunting into alternate pathways.

There were no significant changes in calcium deposition either from low or high cholesterol treatment above control or between low and high cholesterol at both high and low glucose levels. However, significant, albeit small, increases at high versus low glucose were seen in calcification to an equivalent degree at both low and high cholesterol levels (Figure 6.B.). Thus unoxidized cholesterol at both low and high concentrations may exhibit a small influence on calcification at low glucose not demonstrated at high glucose. It would be interesting to see if 25-OHC at high and low glucose would show this difference.

In establishing this *in vitro* model, RPMI, the first chosen medium, did not support nodule formation, whereas DMEM did. There are multiple differences between the two media, however there are some specific differences that may have contributed to this effect that may be worth exploring. DMEM has 4.00 mg/L thiamin HCl whereas RPMI has only 1.00 mg/L. Thiamine (vitamin B1) is important in glucose metabolism as a coenzyme that shunts glycolysis metabolites, specifically 3-phosphoglyceraldehyde into

the pentose phosphate pathway and pyruvate into the Krebs cycle. The inability of RPMI to allow for nodule formation may be due to a lack of adequate vitamin B1 that would allow shunting of glycolysis metabolites. There are several differences in the vitamin levels of these media, with DMEM containing far greater amounts of several vitamins necessary to glucose metabolism than RPMI. For example, there is a greater amount of D-Ca pantothenate, a precursor to Coenzyme A, in DMEM (4.00 mg/L) than in RPMI (0.250 mg/L). Since HMG-CoA is a precursor to mevalonate, which eventually forms cholesterol, this may be important. Exploring the specific differences in vitamins that allow nodule formation may indicate specific pathways used or shunted.

At high glucose, significant increases over control in metabolic (mitochondrial) activity were seen upon addition of cholesterol (see Figure 4.A.). Originally the MTT assay, which measured a dye that is changed in the mitochondria (indicating mitochondrial activity) was simply used to show that the levels of lipids chosen were not toxic to cells. The cells were viable at both low and high cholesterol levels, so neither calcification nor nodule formation was associated with, or could be explained by, increased cell death. There was no significant difference in metabolic activity between low and high cholesterol application. This could be due to low cholesterol producing the maximum metabolic activity observable by this assay. Differences in metabolic/mitochondrial activity may be evident upon further tests with more sensitive assays. There was no significant change in either metabolic activity or calcium deposition between low and high cholesterol treatments. Metabolic activity changes did not appear to explain either the increases in nodule formation demonstrated between low and high cholesterol treatments (see Figure 2.A.), or the lack of significant differences over control

demonstrated in calcium deposition (see Figure 3). The MTT information does not support nor refute the original theory of calcification, which states that increased calcification is due to increased cell death, and is a simple process of ubiquitous phosphate forming apatite upon release of calcium from dead cells.

Our use of MTT demonstrated that the change in nodule formation between low and high glucose at high cholesterol could not be explained by changes in mitochondrial activity. The MTT assay has been used with other cell types to measure proliferation, but was not intended for that purpose in this study. Accelerated proliferation of the normally quiescent VSMCs is known to contribute to atherosclerotic lesion formation. Previous findings gave mixed results when attempting to correlate nodule formation and proliferation activity. Further studies utilizing other indicators more accurate for measuring proliferation such as PCNA stain or BrdU may give better indication of actual proliferation state, and would be of interest.

Unoxidized cholesterol increased the number of nodules formed by vascular smooth muscle cells but did not influence calcification. At high cholesterol, glucose exhibited an influence on the number of nodules formed, with increased glucose somehow limiting the increase in nodule formation due to cholesterol.

7.0 Conclusion

This investigation was undertaken to elucidate some of the molecular mechanisms of atherosclerotic plaque formation and subsequent calcification. The serum factors glucose, cholesterol and triglyceride were evaluated because they may influence these processes due to their elevated levels during diabetes mellitus, a disease state closely associated with increased rates of atherosclerosis.

The results of this study demonstrated that cholesterol increased nodule formation in a dose-dependent fashion, and the rate of nodule formation at both high and low glucose. Triglyceride had these same effects, but to a much smaller magnitude. Cholesterol did not cause significant change in calcium deposition at either low or high glucose. Either a better calcium assay, such as Ca^{45}, or normalizing the calcium deposition to the amount of protein, as was done by other groups, may reveal results more closely resembling that of other groups using 25-OHC [6, 7, 13]. The increased nodule formation and rate of nodule formation observed at high cholesterol was much greater at low glucose, revealing that glucose influenced the lipid effects on smooth muscle cell nodule formation. The number of nodules formed was not determined to be an indication of the level of calcification in cell culture.

These results suggest that atherosclerotic lesion formation and calcification may occur through different mechanisms. Unoxidized cholesterol may contribute to early atherosclerotic lesion formation *in vivo*, as indicated by increased nodule formation *in vitro*. Perhaps oxidized cholesterol, in the form of 25-OHC, is required for increased calcification of atherosclerotic plaques. The oxidized 25-OHC may act through vitamin D receptors to increase calcification, as has been hypothesized by Demer et al, whereas

unoxidized cholesterol may act through scavenger receptors found on SMCs to increase atherosclerotic lesions [6, 7,13]. The increase in nodule formation at low versus high levels of glucose implies that starvation for glucose states, such as those found during insulin resistance in diabetes mellitus, may modulate lipid effects on vascular cells. Future studies should be conducted using this model to determine whether 25-hydroxycholesterol, versus unoxidized cholesterol, increases calcium deposition, thereby suggesting that only oxidized cholesterol influences the later atherosclerotic mechanism of calcification. Testing unoxidized cholesterol and 25-OHC in the presence and absence of vitamin D receptor anti-bodies may shed light on whether these receptors are required for the increased calcification seen previously with 25-OHC. The glucose influence on the lipid effects on nodule formation by vascular cells emphasizes the need for exploring this effect when experimenting with those factors affecting cholesterol effects on vascular cells.

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