

# Natural nutrient mixture effectively reduces collagen matrix contraction driven by human uterine smooth muscle cells

Vadim Ivanov, M. Waheed Roomi, Tatiana Kalinovsky, Aleksandra Niedzwiecki and Matthias Rath

Matthias Rath Research Institute, Santa Clara, California, USA

## Abstract

**Aim:** Abnormal uterine myometrial contractility causes preterm delivery, contributing to perinatal morbidity and mortality. Disturbances in hormonal regulation and inflammation-related processes have been attributed a role in the pathophysiological mechanisms of uterine contractility. We investigated the effects of natural nutrients on uterine tissue contractility *in vitro*.

**Methods:** We used an *in vitro* model of collagen I gel contraction driven by embedded cultured human uterine smooth muscle cells (SMC). The effects of tested compounds were evaluated using their capacity to affect gel contraction (measured by reduction in gel area during 24-h incubation in serum free medium). Cellular expression of matrix metalloproteinases (MMP) was followed by gel zymography.

**Results:** Collagen gel contraction driven by uterine SMC was significantly stimulated by potassium chloride, pituitary hormone oxytocin and by inflammatory cytokine  $\alpha$ -tumor necrosis factor (TNF- $\alpha$ ). Accelerated gel contraction was accompanied by elevated secretion of MMP-2 and MMP-9 into cell culture media. Among a variety of purified bioflavonoids and polyphenol-enriched plant extracts tested for their ability to counteract uterine SMC-dependent collagen gel contraction, the strongest effects were demonstrated by epigallocatechin gallate and green tea leaf extract, respectively. The addition of ascorbic acid and the amino acids lysine, arginine, cysteine and proline to green tea extract further increased its effectiveness. A reduction in gel contraction correlated with decreased MMP expression.

**Conclusion:** Based on these findings, we found that nutrients can effectively counteract uterine myometrial contraction and MMP activity *in vitro*, suggesting that pathophysiological mechanisms of abnormal uterine myometrial contractility can be counteracted by a combination of naturally occurring nutrients. These mechanisms might involve extracellular matrix remodeling.

**Key words:** bioflavonoids, collagen gel contraction, human uterine smooth muscle cell, nutrients, premature labor.

## Introduction

Abnormal uterine myometrial contractility causes preterm delivery, contributing to perinatal morbidity and mortality.<sup>1</sup> Approximately 31–50% of preterm births

are caused by spontaneous preterm labor. In a 20-year follow-up study, low birthweight infants (<1500 g) were more likely to experience neurosensory deficits (blindness, deafness, cerebral palsy), a lower IQ, and subnormal height.<sup>2</sup> Studies have linked disturbances in

Received: June 6 2005.

Accepted: October 28 2005.

Reprint request to: Tatiana Kalinovsky, 13980W. Bell Road, Ste. 11, PMB #125, Surprise, AZ 85374, USA. Email: t.kalinovsky@drath.com

hormonal regulation and inflammation-related processes with premature uterine contraction.<sup>3,4</sup>

Attempts to delay the onset or to inhibit preterm labor using drugs have been unsuccessful. Previously, the major emphasis was on the development of agonists of the uterorelaxant pathway or antagonists of the uterotonic pathways.  $\beta$ 2 adrenergic agonists have been widely used clinically, but are highly correlated with adverse effects; delay in delivery did not achieve objective perinatal benefit as measured by mortality or morbidity. Cyclooxygenase-2 (COX-2) inhibitors and human chorionic gonadotropin (hCG) have been suggested as new strategies for myometrial relaxation.<sup>1</sup> Several clinical studies indicate that supplementation with vitamin C and E might exert a protective effect against premature birth.<sup>5-7</sup> Panburana *et al.* reported that antioxidant vitamin C and E levels were significantly decreased in pre-eclamptic women compared with normal pregnant women and lipid peroxide levels were significantly higher in the pre-eclamptic group.<sup>8</sup>

Oxytocin (including estradiol-stimulated oxytocin receptor expression), vasopressin and endothelin-1 (ET-1) are critical to hormonal regulation of uterine contraction, and have distinct receptor-mediated systems of interaction with uterine smooth muscle cells (SMC).<sup>9-12</sup> It is now becoming evident that infection and inflammation are involved in the pathogenesis of preterm labor and delivery, and a link between TNF- $\alpha$  and interleukin (IL)-1 $\beta$  and premature childbirth has been proposed by Arntzen *et al.*<sup>13</sup> In addition, there is evidence that matrix metalloproteinases (MMP) are involved in stimulation of uterine tissue contraction. Fetal membrane MMP-9 levels have been reported to increase during active labor prior to delivery.<sup>3,14-18</sup>

Dallot *et al.* demonstrated that cultured uterine SMC have the capacity to contract in response to an agonist previously reported to induce a potent *in vitro* contractile response in intact myometrial strips; ET-1-stimulated contraction of cultured uterine SMC in a collagen lattice in a dose-dependent manner.<sup>19</sup> The culture system preserved the myometrial cell responses to ET-1 in terms of contractility and receptors involved in this effect, implying that cultured myometrial cells retain the normal physiological properties that occur *in vivo* in fully differentiated SMC in a normal environment. Contractile responses to oxytocin and prostaglandin are retained by uterine SMC after subculturing. This model provides for identifying potential regulatory pathways and for studying the action of a variety

of signaling factors that modulate both normal and pathological uterine activity.

In a previous study, we found that a variety of bioflavonoids were effective in reversing angiotensin II-induced aortic SMC contraction when tested in a collagen I gel lattice (Ivanov *et al.* unpubl. data, 2003). The potency of bioflavonoid activity was found to depend on structure. In this study we used the *in vitro* model of collagen I gel contraction driven by embedded cultured human uterine SMC to study the effect of natural nutrients on uterine contraction induced by hormones and inflammatory agents.

## Materials and Methods

### Materials

Tissue culture plastics were obtained from Becton Dickinson (Franklin Lakes, NJ, USA). Tissue culture supplies (growth media, antibiotics and trypsin-EDTA) were obtained from Life Technologies. Fetal bovine serum (FBS) was from BioWhittaker (Walkersville, MD, USA). L-ascorbic acid, bovine serum albumin (BSA; fraction V), and other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

### Cell culture

Human uterine SMC (obtained from Clonetics, San Diego, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, penicillin (100  $\mu$ g/mL) and streptomycin (100  $\mu$ g/mL) at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>, and were split 1:3-1:5 upon reaching the confluence. Uterine smooth muscle cell (USMC) at passages 6-8 were used in experiments.

### Collagen contraction

Collagen gel preparation was carried out as described by Bogatkevich *et al.*<sup>20</sup> Collagen lattices were prepared using type I collagen from rat-tail tendon (BD Bioscience, Bedford, MA, USA). ASMC ( $2.5 \times 10^5$  cells/mL final concentration) suspension was mixed with an equal volume of collagen solution (1.25 mg/mL of collagen final concentration) and aliquoted by 300  $\mu$ l into 24-well plates pretreated with 2 mg/mL BSA and dried before use. Collagen lattices were polymerized for 45 min in a humidified 10% CO<sub>2</sub> atmosphere at 37°C. To initiate collagen gel contraction, tested compounds in serum-free DMEM were added to the wells and the gels were set afloat by gentle tapping. Digital photographs were taken of the gels after a 24-h period of incubation at 37°C. The degree of collagen gel



contraction was determined after 24 h, by measuring the area of contracted gel using Scion Image software (complements of Scion, Frederick, MD, USA). Results are expressed as a percentage of the control gel area (DMEM) and presented as mean  $\pm$  SD from three or more replicates. Gel with embedded uterine SMC contracts spontaneously in DMEM in the absence of additional stimuli.

### Gelatinase zymography

MMP expression in condition media was determined using gelatinase zymography. Gelatinase zymography was performed in 10% Novex precast SDS-polyacrylamide gel (Invitrogen, Carlsbad, CA, USA) in the presence of 0.1% gelatin under non-reduced conditions. Culture media (20  $\mu$ L) mixed with sample buffer was loaded and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with Tris glycine SDS buffer, as described by the manufacturer. Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove the SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl<sub>2</sub> at pH 8.0 and stained with 0.5% Coomassie Blue R250 (AnaSpec, San Jose, CA, USA) in 50% methanol and 10% glacial acetic acid for 30 min and destained. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

### Cell proliferation/cytotoxicity: MTT assay

Cell proliferation was evaluated using MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (3-[4,5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide) to a blue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. The cells were washed with phosphate buffered saline (PBS) and 500  $\mu$ L of MTT (#M-2128; Sigma-Aldrich) 0.5 mg/mL in media was added to each well. After MTT addition (0.5 mg/mL), the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 mL dimethylsulfoxide (DMSO), and absorbance was measured at 570 nm in a Bio Spec 1601, Shimadzu spectrometer. The OD<sub>570</sub> of the DMSO solution in each

well was considered to be proportional to the number of cells. The OD<sub>570</sub> of the control (treatment without supplement) was considered to be 100%.

### Cell morphology

Morphology of uterine SMC cultured in test concentrations of nutrient mixture (NM) were evaluated using hematoxylin and eosin (H&E) staining and observed and photographed using microscopy.

### Composition of the nutrient mixture

The stock solution of the NM used for testing was composed of the following: vitamin C (as ascorbic acid and as Mg, Ca and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract 1000 mg (green tea extract derived from green tea leaves was obtained from US Pharma Laboratory, Somerset, NJ, USA). The certificate of analysis indicates the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin gallate (EGCG) 35%, caffeine 1.0%; selenium 30 mg; copper 2 mg; manganese 1 mg.

### Statistical analysis

Results were expressed as mean  $\pm$  SD for the groups. Data were analyzed using independent sample *t*-tests. The correlation between MMP-9 bands densitometer and gel contraction was also determined.

## Results

### USMC-mediated contraction of collagen I gel

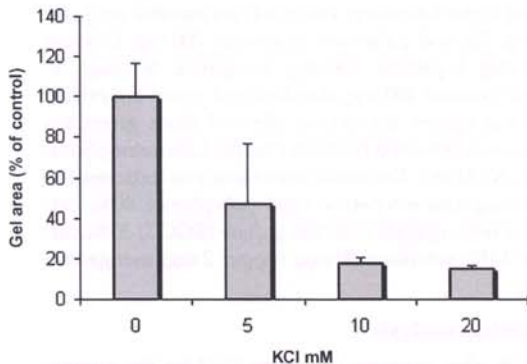
*Dose-dependent potassium chloride collagen gel contraction*  
Collagen gel contraction driven by uterine SMC was significantly stimulated by potassium chloride in a dose-dependent manner (Fig. 1).

### Effect of oxytocin and nutrient mixture on USMC-mediated collagen I gel contraction

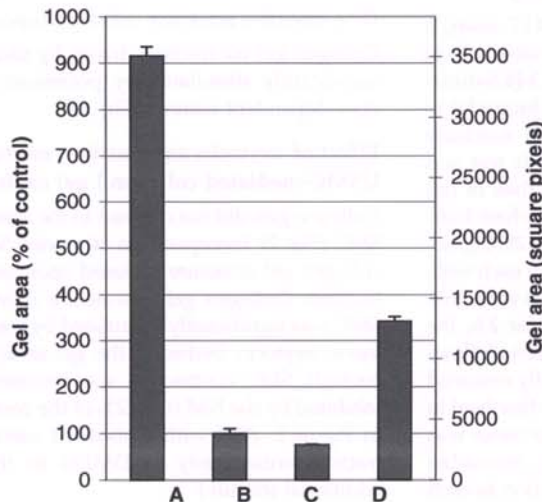
Collagen gels did not contract in the absence of uterine SMC (Fig. 2). Incorporation of uterine SMC into a 3-D collagen gel structure initiated spontaneous gel contraction. Collagen gel contraction driven by uterine SMC was significantly stimulated by the pituitary hormone oxytocin (reducing the gel area to 68% of the control); SMC contraction was reversed and further inhibited by the NM (to 342% of the control) as shown in Figure 2. (Gel with embedded uterine SMC contracts spontaneously in DMEM in the absence of additional stimuli.)

### Effect of different classes of bioflavonoids on USMC-mediated collagen I gel contraction

Among a variety of purified bioflavonoids tested for their ability to counteract uterine SMC-dependent collagen gel contraction, the strongest effects were demonstrated by EGCG (229% of control,  $P < 0.0001$ ), followed by genistein (163%,  $P = 0.0008$ ), quercetin (153%,  $P = 0.002$ ), resveratrol (120%,  $P = 0.10$ ), and rutin (113%,  $P = 0.17$ ; Fig. 3). Among polyphenol-enriched plant extracts (at a concentration of 20  $\mu\text{g}/$



**Figure 1** Effect of potassium chloride (KCl) on uterine smooth muscle cell-mediated contraction of collagen I gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates.

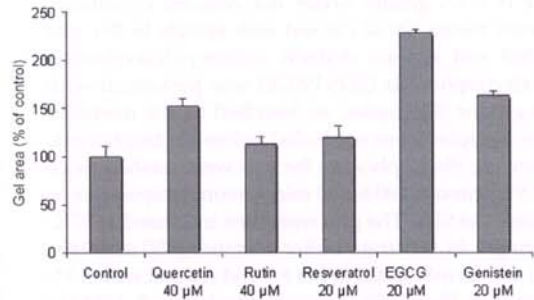


**Figure 2** Effect of oxytocin and the nutrient mixture on uterine smooth muscle cell (SMC)-mediated contraction of collagen I gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates.

mL), the strongest effects were demonstrated by green tea leaf extract (521% of control,  $P < 0.0001$ ), followed by pine bark extract (305% of control,  $P < 0.0001$ ; Fig. 4).

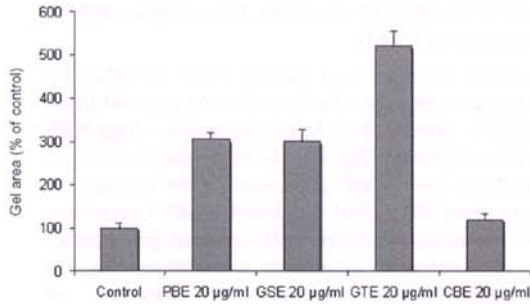
### Effect of nutrients USMC-mediated contraction of collagen gel

Individual nutrients tested had no significant effect on uterine SMC contraction except EGCG (a derivative of green tea extract) and green tea extract. EGCG reversed contraction to 124% of control ( $P = 0.0080$ ) and green tea extract increased the gel area by 252% of control ( $P = 0.0002$ ). The addition of ascorbic acid and

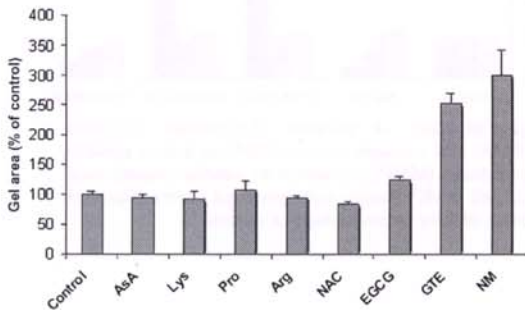


**Figure 3** Effects of purified polyphenols on uterine smooth muscle cell-mediated contraction of collagen I gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates. EGCG, epigallocatechin gallate.





**Figure 4** Effect of plant-derived polyphenol extracts on uterine smooth muscle cell-mediated contraction of collagen I gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates. CBE, citrus bioflavonoid extract; GSE, grape seed extract; GTE, green tea extract; PBE, pine bark extract. All extracts used at a concentration of 20 µg/mL of total phenols.

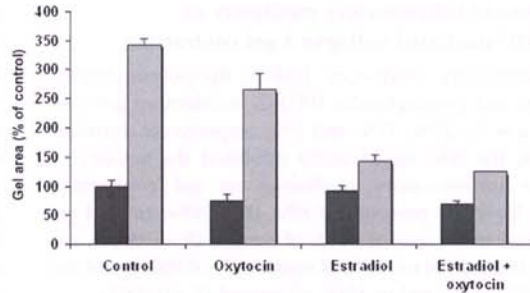


**Figure 5** Effects of nutrient mixture components on uterine smooth muscle cell-mediated contraction of collagen I gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates. Arg, arginine 30 µM; AsA, ascorbic acid 100 µM; EGCG, epigallocatechin gallate 15 µM; GTE, green tea extract 20 µg/mL; Lys, lysine 100 µM; NAC, N-acetyl cysteine 25 µM; NM, nutrient mixture 100 µg/mL; Pro, proline 100 µM.

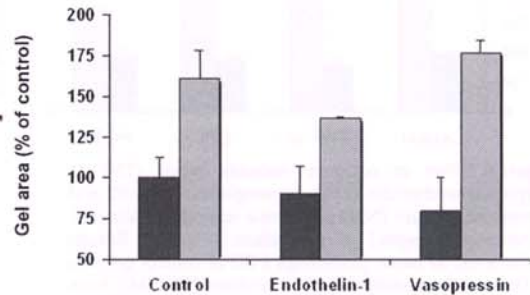
amino acids to green tea extract further increased its effectiveness (to 300% of control,  $P = 0.0013$ ; Fig. 5).

#### Effects of hormones on USMC-mediated contraction of collagen I gel

Collagen gel contraction driven by uterine SMC was stimulated by oxytocin, estradiol, and oxytocin



**Figure 6** Effect of oxytocin (10 nM), estradiol (100 nM) and nutrient mixture (NM) on uterine smooth muscle cell-mediated contraction of collagen gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates. (■) No NM, (□) NM 100 µg/mL.

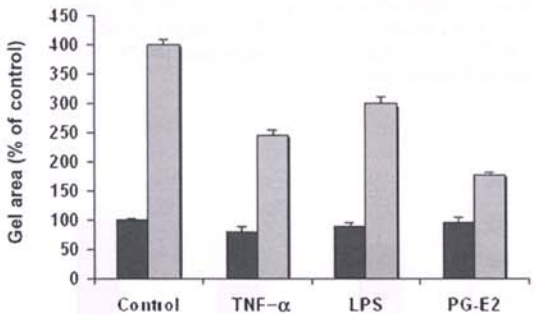


**Figure 7** Effect of endothelin-1 (100 ng/mL), vasopressin (10 nM) and nutrient mixture (NM) on uterine smooth muscle cell-mediated contraction of collagen I gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates. (■) No NM, (□) NM 100 µg/mL.

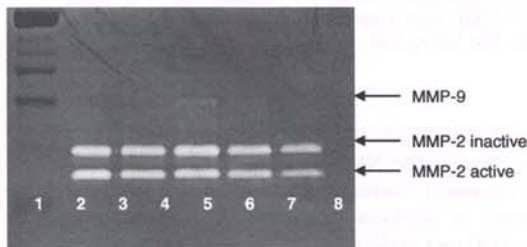
+ estradiol to 75.6%, 92% and 70% of control, respectively (Fig. 6). The NM significantly inhibited uterine SMC-mediated contraction of collagen I gel in the presence of oxytocin (by 350% of oxytocin alone,  $P = 0.0004$ ) and in the presence of estradiol (by 155% of estradiol alone,  $P = 0.00370$ ). ET-1 and vasopressin also stimulated uterine SMC-mediated contraction of collagen I gel by 10% and 20%, respectively (Fig. 7). NM significantly reversed the action of these hormones and further relaxed the gel matrix by 152% ( $P = 0.0087$ ) and 221% ( $P = 0.0014$ ), respectively.

### Effects of inflammatory mediators on USMC-mediated collagen I gel contraction

Inflammatory mediators TNF- $\alpha$ , lipopolysaccharide (LPS) and prostaglandin (PG)-E2 accelerated gel contraction by 21%, 11% and 5%, respectively. Furthermore, the NM significantly inhibited the activity of these inflammatory mediators on gel contraction (Fig. 8). In the presence of NM, the TNF- $\alpha$ -treated gel relaxed to an area of 31% of control ( $P < 0.0001$ ), the LPS-treated gel to 33% of control ( $P < 0.0001$ ), and the PG-E2-treated gel to 124% of control ( $P = 0.0001$ ).



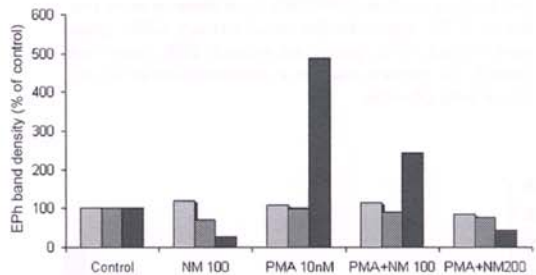
**Figure 8** Effect of  $\alpha$ -tumor necrosis factor (TNF- $\alpha$ ), lipopolysaccharides (LPS), prostaglandin (PG)-E2 and nutrient mixture (NM) on uterine smooth muscle cell-mediated collagen I gel contraction: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates. (■) No NM, (□) NM 100  $\mu$ g/mL.



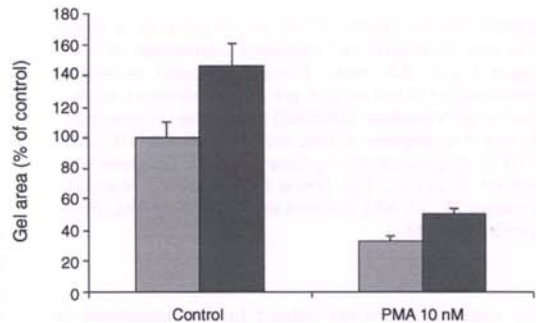
**Figure 9** Effect of phorbol 12-myristate 13-acetate (PMA) and nutrient mixture (NM) on matrix metalloproteinase (MMP) expression by uterine smooth muscle cell during collagen I gel contraction: gelatinase zymography. 1, molecular weight markers; 2, control; 3, NM 100  $\mu$ g/mL; 4, PMA 10 nM; 5, PMA + NM 100  $\mu$ g/mL; 6, PMA + NM 200  $\mu$ g/mL.

### Effect of phorbol 12-myristate 13-acetate on MMP expression of USMC

The direct stimulation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) resulted in an increase in uterine SMC MMP-9 secretion (Figs 9,10) and gel contraction (Fig. 11). The NM reduced both gel contraction and MMP expression; NM 200  $\mu$ g/mL greater than 100  $\mu$ g/mL inhibited MMP-9 secretion. PMA treatment of uterine SMC-induced gel contraction demonstrated significant correlation (Fig. 12) with PMA-induced uterine SMC secretion of MMP-9 ( $r = -0.9118$ ,  $P = 0.0016$ ), indicating possible



**Figure 10** Effect of phorbol 12-myristate 13-acetate (PMA) and nutrient mixture (NM) on matrix metalloproteinase (MMP) expression by uterine smooth muscle cell (SMC) during collagen I gel contraction: Eph band density as percentage of control.



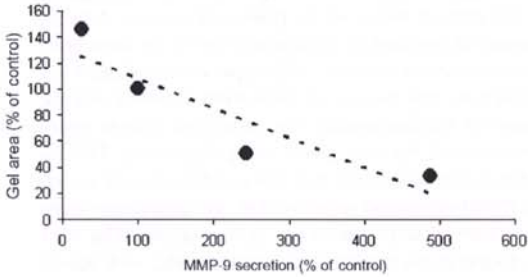
**Figure 11** Effect of phorbol 12-myristate 13-acetate (PMA) and nutrient mixture (NM) on uterine smooth muscle cell-mediated contraction of collagen I gel: 24-h assay. Results presented as mean percentage  $\pm$  SD of control gel area (Dulbecco's modified Eagle's medium [DMEM]) from three or more replicates. (■) No NM, (□) NM 100  $\mu$ g/mL.



involvement of extracellular matrix remodeling in gel contraction by MMP activity. We found no correlation between MMP-2 secretion and gel contraction.

**Effect of the nutrient mixture on USMC viability: MTT assay**

NM showed no significant toxicity to the uterine SMC even at 1000 µg/mL, as shown in Figure 13.



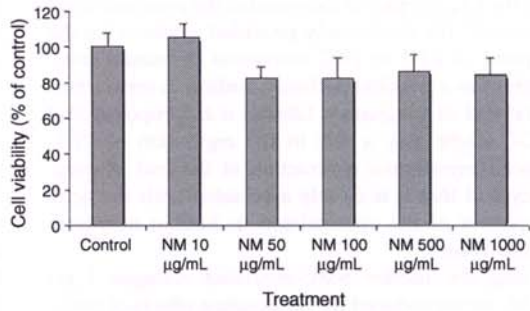
**Figure 12** Correlation analysis for matrix metalloproteinase-9 (MMP-9) expression and gel contraction by uterine smooth muscle cell under phorbol 12-myristate 13-acetate (PMA) treatment:  $r = -0.9118$ ,  $P = 0.0016$ .

**Effect of the nutrient mixture on USMC morphology: H&E stains**

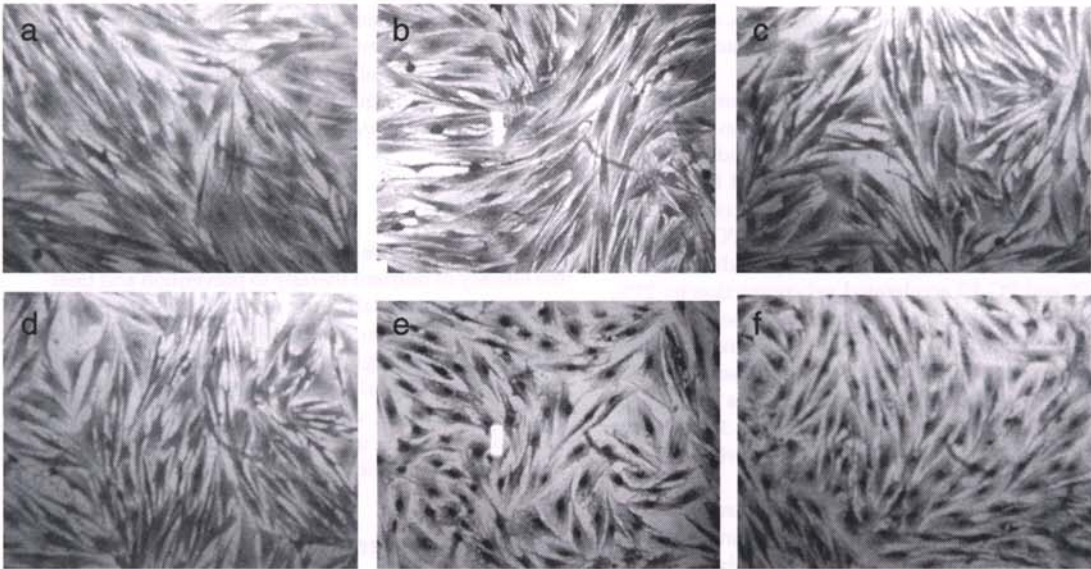
H&E staining showed no morphological changes, even at the highest concentration of NM (Fig. 14a–f).

**Discussion**

At near-term, the uterine myometrium becomes extremely sensitive to oxytocin, the strongest utero-tonic agent known. Ovarian steroids, estrogen and



**Figure 13** Effect of nutrient mixture (NM) on uterine smooth muscle cell viability: 24-h MTT assay.



**Figure 14** Effect of nutrient mixture (NM) on uterine smooth muscle cell morphology. (a) Control, (b) NM 10 µg/mL, (c) NM 50 µg/mL, (d) NM 100 µg/mL, (e) NM 500 µg/mL, (f) NM 1000 µg/mL (H&E staining).

progesterone have been reported to be involved in the regulation of oxytocin function. Murata *et al.* reported that estrogen stimulated the number of uterine oxytocin binding sites, while adding progesterone inhibited the estrogen stimulation effect.<sup>11</sup> The maternal blood concentration of ET-1 increases during pregnancy and peaks at term. Human uterine muscle is sensitive to ET-1 and ET-1 induced uterine contraction is markedly increased at the end of pregnancy. In rats, uterine contractile responsiveness to ET-1 is elevated during labor and diminished postpartum. Of the two types of endothelin receptors in human myometrial tissues, only the ET<sub>A</sub> receptor is increased in the pregnant myometrium.<sup>21</sup> The Eude study provided evidence for the coupling of ET-1 to PKC activation in human myometrium as a possible pathway leading to contraction at the end of pregnancy. Liberto *et al.* proposed that PKC- $\zeta$  might play a role in the regulation of ET-1-induced myometrial contraction at the end of pregnancy and that it is closely associated with the actin component of the cytoskeleton in human near-term myometrium.<sup>4</sup>

Using the uterine SMC-mediated collagen I gel model, we reproduced the stimulating effects of major hormones (oxytocin, vasopressin, estradiol and ET-1) involved in human uterine myometrial contractility, which can lead to preterm delivery. Collagen gel contraction driven by uterine SMC was stimulated by oxytocin, estradiol, ET-1 and vasopressin, and significantly inhibited by the NM of ascorbic acid, proline, lysine, arginine and green tea extract.

It is now evident that infection and inflammation are involved in the pathogenesis of preterm labor and delivery<sup>22</sup> and a link between TNF- $\alpha$  and IL-1 $\beta$  and premature childbirth has been proposed by Arntzen *et al.*<sup>13</sup> Using the uterine SMC-mediated collagen I gel model, we found that TNF- $\alpha$ , LPS and PG-E2 stimulated SMC contraction. Inflammatory mediators TNF- $\alpha$ , LPS and PG-E2 accelerated gel contraction by 21%, 11% and 5%, respectively. Furthermore, the NM significantly inhibited the activity of these inflammatory mediators on gel contraction. In the presence of NM, the TNF- $\alpha$ -treated gel relaxed to an area of 311% of control ( $P < 0.0001$ ), the LPS-treated gel to 337% of control ( $P < 0.0001$ ), and the PG-E2-treated gel to 124% of control ( $P = 0.0001$ ).

Recent evidence indicates that aPKC are important components of the TNF- $\alpha$ /IL-1 $\beta$  signaling pathway that controls nuclear factor  $\kappa\beta$  (NF- $\kappa\beta$ ) activation<sup>23</sup> and that targeted disruption of the gene of PKC- $\zeta$  results in the impairment of the NF- $\kappa\beta$  pathway.<sup>24</sup> Apparently

both stimuli could involve PKC and downstream NF- $\kappa\beta$ -mediated mechanisms, thus implying the common points in different stimuli action. In our study, the direct stimulation of PKC by PMA resulted in a corresponding increase in MMP-9 secretion. Uterine SMC MMP-9 secretion and gel contraction induced by PMA treatment demonstrated significant correlation, suggesting possible involvement of extracellular matrix remodeling in gel contraction by MMP activity.

Among polyphenol-enriched plant extracts tested, the strongest relaxant effects were observed with green tea leaf extract, followed by pine bark extract. Among a variety of purified bioflavonoids tested for their ability to counteract uterine SMC-dependent collagen gel contraction, the degree of relaxation differed among classes of bioflavonoids; the strongest effects were demonstrated by the green tea polyphenol, EGCG. Additionally, we found that the combination of nutrients (NM) enhanced uterine SMC gel relaxation over that achieved with green tea extract alone. The NM was found to be non-toxic to uterine SMC well above the test range of the contraction studies. Furthermore, the effective concentration of the NM (100  $\mu\text{g}/\text{mL}$  to 200  $\mu\text{g}/\text{mL}$ ) in our study corresponds to the reference range of amino acid plasma values for healthy human individuals.<sup>25</sup> Amino acid plasma in this range responds positively to oral supplementation.<sup>26</sup> EGCG plasma concentration has been shown to reach 3  $\mu\text{M}$  secondary to supplementation with a single oral dose in healthy volunteers,<sup>27</sup> which corresponds approximately to 100  $\mu\text{g}/\text{mL}$  of the NM used in our study. Thus, we conclude that the biological effects described in this study are relevant to physiological conditions.

## Conclusion

While clinical studies are necessary to better determine the efficacy of nutrient therapy in prevention and treatment of premature labor, the results of this study suggest the combination of naturally occurring nutrients of lysine, proline, arginine, ascorbic acid and green tea has potential clinical use in counteracting abnormal uterine myometrial contractility, secondary to effectively counteracting uterine SMC-induced gel contraction and MMP activity *in vitro*.

## Acknowledgments

Funding was provided by the Matthias Rath Research Institute BV.



## References

- Slattery MM, Morrison JJ. Preterm delivery. *Lancet* 2002; **360**: 1489–1497.
- Hack M, Flannery DJ, Schluchter M *et al*. Outcomes in young adulthood for very-low-birth-weight infants. *N Engl J Med* 2002; **346**: 149–157.
- Maymon E, Romero R, Pacora P *et al*. Evidence of *in vivo* differential bioavailability of the active forms of matrix metalloproteinases 9 and 2 in parturition, spontaneous rupture of membranes, and intra-amniotic infection. *Am J Obstet Gynecol* 2000; **183**: 887–894.
- Di Liberto G, Dallot E, Eude-Le Parco I, Cabrol D, Ferre F, Breuille-Fouche M. A critical role for PKC zeta in endothelin-1-induced uterine contractions at the end of pregnancy. *Am J Physiol Cell Physiol* 2003; **285**: C599–C607.
- Siega-Riz AM, Promislow JH, Savitz DA, Thorp JM Jr, McDonald T. Vitamin C intake and the risk of preterm delivery. *Am J Obstet Gynecol* 2003; **189**: 519–525.
- Woods JR Jr, Plessinger MA, Miller RK. Vitamin C and E: missing links in preventing preterm premature rupture of membranes. *Am J Obstet Gynecol* 2001; **185**: 5–10.
- Romero R, Chalworapongsa T, Espinoza J. Micronutrients and intrauterine infection, preterm birth and the fetal inflammatory response syndrome. *J Nutr* 2003; **133**: 1668S–1673S.
- Panburana P, Phuapradit W, Puchaiwatananon O. Antioxidant nutrients and lipid peroxide levels in Thai preeclamptic pregnant women. *J Obstet Gynaecol Res* 2000; **26**: 377–381.
- Zingg HH, Laporte SA. The oxytocin receptor. *Trends Endocrinol Metab* 2003; **14**: 222–227.
- Richter ON, Kubler K, Schmolling J *et al*. Oxytocin receptor gene expression of estrogen-stimulated human myometrium in extracorporeally perfused non-pregnant uteri. *Mol Hum Reprod* 2004; **10**: 339–346.
- Murata T, Murata E, Liu C-X, Narita K, Honda K, Higuchi T. Oxytocin receptor gene expression in rat uterus: regulation by ovarian steroids. *J Endocrinol* 2000; **166**: 45–52.
- Tamura M, Deb S, Sebastian S, Okamura K, Bulun SE. Estrogen up-regulates cyclooxygenase-2 via estrogen receptor in human uterine microvascular endothelial cells. *Fertil Steril* 2004; **81**: 135–136.
- Arntzen KJ, Kjollesdal AM, Halgunset J, Vatten L, Austgulen R. TNF, IL-1, IL-6, IL-8 and soluble TNF receptors in relation to chorioamnionitis and premature labor. *J Perinat Med* 1998; **26**: 17–26.
- Roh CR, Oh WJ, Yoon BK, Lee JH. Up-regulation of matrix metalloproteinase-9 in human myometrium during labor; a cytokine-mediated process in uterine smooth muscle cells. *Mol Hum Reprod* 2000; **6**: 96–102.
- Vadillo-Ortega F, Gonzalea-Avila G, Furth EE *et al*. 92-kd type collagenase (matrix metalloproteinase-9) activity in human amniochorion increases with labor. *Am J Pathol* 1991; **146**: 148–156.
- McLaren J, Taylor DJ, Bell SC. Increased concentration of pro-matrix metalloproteinase 9 in term fetal membranes overlying the cervix before labor: implications for membrane remodeling and rupture. *Am J Obstet Gynecol* 2000; **182**: 409–416.
- Xu P, Alfaidy N, Challis JRG. Expression of matrix metalloproteinase (MMP-2 and MMP-9) in human placenta and fetal membranes in relation to preterm and term labor. *J Clin Endocrinol Metab* 2002; **87**: 1353–1361.
- Goldman S, Weiss A, Eyal Y, Shaker E. Differential activity of the gelatinase (matrix metalloproteinases 2 and 9) in the fetal membranes and deciduas, associated with labour. *Mol Hum Reprod* 2003; **9**: 367–373.
- Dallot E, Pouchelet M, Gouhier N, Cabrol D, Ferre F, Breuille-Fouche M. Contraction of human uterine smooth muscle cells after stimulation with endothelin-1. *Biol Reprod* 2003; **68**: 937–942.
- Bogatkevich GS, Tourkina E, Silver RM *et al*. Thrombin differentiates normal lung fibroblasts to a myofibroblast phenotype via the proteolytically activated receptor-1 and a protein kinase C-dependent pathway. *J Biol Chem* 2001; **276**: 45184–45192.
- Eude I, Dallot E, Ferre F, Breuille-Fouche M. Protein kinase C alpha is required for endothelin-1-induced proliferation of human myometrial cells. *Biol Reprod* 2002; **66**: 44–49.
- Keski-Nisula LT, Aalto ML, Kirkinen PP, Kosma VM, Heinonen ST. Myometrial inflammation in human delivery and its association with labor and infection. *Am J Clin Pathol* 2003; **120**: 217–224.
- Diaz-Meco MT, Dominguez I, Sanz I *et al*.  $\zeta$ PKC induces phosphorylation and inactivation of I $\kappa$ B- $\alpha$  *in vitro*. *EMBO J* 1994; **13**: 2842–2848.
- Leitges M, Sanz L, Martin P *et al*. Targeted disruption of the  $\zeta$ PKC gene results in the impairment of the NF- $\kappa$ B pathway. *Mol Cell* 2001; **8**: 771–780.
- National Institute of Health. [Cited October 2004]. Available from URL: <http://www.nlm.nih.gov/medlineplus/ency/article/003361.htm>.
- Meredith CN, Wen ZM, Bier DM, Matthews DE, Young VR. Lysine kinetics at graded lysine intakes in young men. *Am J Clin Nutr* 1986; **43**: 787–794.
- Ullmann U, Haller J, Decourt JP *et al*. A single ascending dose study of epigallocatechin gallate in healthy volunteers. *J Int Med Res* 2003; **31**: 88–101.