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# Human umbilical cord expresses several vasoactive peptides involved in the local regulation of vascular tone: protein and gene expression of Orphanin, Oxytocin, ANP, eNOS and iNOS

**Annamaria Mauro, Maria Buscemi, Salvatore Provenzano, Aldo Gerbino**

BioNeC: Dipartimento di Biomedicina Sperimentale e Neuroscienze Cliniche, Sezione di Istologia ed Embriologia "Arcangelo Pasqualino di Marineo", Facoltà di Medicina e Chirurgia, Università degli Studi di Palermo, Italy

**Abstract:** Full-term human umbilical cord contains three blood vessels: two arteries coiled around a vein and surrounded by Wharton's jelly, a mucous tissue with few mesenchymal stromal cells and abundant extracellular matrix. Umbilical vessels lack innervations, thus endothelial cells must play a role in the control of blood flow. The aim of this study was to investigate in human umbilical cord the expression of five peptides that could be involved in the regulation of vascular tone: Orphanin FQ, Oxytocin, Atrial Natriuretic Peptide (ANP), endothelial Nitric Oxide Synthase (eNOS) and inducible Nitric Oxide Synthase (iNOS). The expression of these molecules in full-term human umbilical cord was investigated through immunohistochemistry and RT-PCR. Immunoreactivity for Orphanin FQ was detected in Wharton's jelly, vessel musculature and endothelium; Oxytocin, ANP and eNOS were expressed by the umbilical epithelium, Wharton's jelly and endothelium, whereas iNOS only by endothelial cells. RT-PCR analysis showed transcriptional expression of Oxytocin, ANP and eNOS mRNAs. The presence of Orphanin, Oxytocin, ANP, eNOS and iNOS proteins was identified in the human umbilical cord. mRNA expression for Oxytocin, ANP and eNOS suggest that these molecules are synthesized by umbilical cord cells themselves. The expression of these vasoactive molecules could be part of a general mechanism locally regulating vascular tone. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 2, pp. X–X*)

**Key words**: human umbilical cord, Orphanin, Oxytocin, ANP, eNOS, iNOS

## Introduction

The umbilical cord is the organ connecting the fetus to the placenta, responsible for the transport of blood and nourishment to the fetus. Three blood vessels can be identified in the full-term human umbilical cord (Figure 1); two arteries coiled around a vein: these vessels are surrounded by Wharton's jelly, a mucous tissue composed of a few mesenchymal stromal cells and an abundant extracellular matrix rich in water, glycosaminoglycans, proteoglycans and collagen fibers [1, 2]. Most current knowledge of the ultrastructure of the umbilical cord derives from electron microscopy studies carried out in the 1970s.

Human umbilical cord epithelium was described for the first time by Hoyes; its structure is very similar to that of the fetal epidermis with the exception that it is not keratinized. It is composed of one to five layers of flattened cells, with some of the superficial cells lacking nuclei and organelles, and their cytoplasm containing a loose meshwork of filamentous material [3].

The endothelium of umbilical blood vessels (both vein and arteries) consists of a single layer of endothelial cells, whose cytoplasm is rich in mitochondria, endoplasmic reticulum and Golgi apparatus, and contains large deposits of glycogen and Weibel & Palade bodies [4].

**Correspondence address:** A. Gerbino, BioNec,
Via del Vespro 129, 90127 Palermo, Italy;
tel.: (+ 39 091) 655 35 84; fax: (+ 39 091) 655 35 86;
e-mail: aldogerbino@tiscali.it

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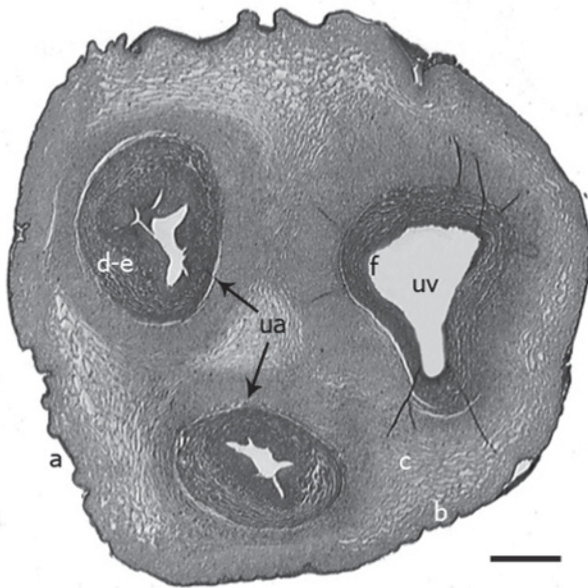


**Figure 1.** Azan staining of a section of full-term human umbilical cord. a — amniotic epithelium; b — sub-amniotic Wharton's jelly; c — inner Wharton's jelly; d–e — circular and longitudinal layers of vascular musculature; f — vascular endothelium; ua — umbilical artery; uv — umbilical vein, bar 1,8 mm

Parry described the morphology of Wharton's jelly mesenchymal cells: these cells are elongated with thin, long processes extending from the cell body; they have a well developed rough endoplasmic reticulum and Golgi apparatus, indicating an intense activity of protein synthesis and secretion [5]. Wharton's jelly mesenchymal cells are similar both to fibroblasts and smooth muscle cells and are therefore considered as myo-fibroblasts [6, 7]. These stromal cells express both muscle-specific contractile proteins, such as actin, myosin, desmin and $\alpha$-smooth muscle actin [6, 8], and mesenchymal stem cell (MSCs) markers, such as CD105, CD73 and CD90, while they do not express hematopoietic stem cell markers [9]. Based on this evidence, it is realistic to assume that umbilical cord stromal cells contain more than one set of stem cell population; in agreement with this hypothesis, umbilical cord stromal cells have been successfully differentiated into adipocytes, chondrocytes, osteocytes, cardiomyocytes and skeletal myocytes *in vitro* [10–12], as well as partially differentiated into neurons, glial cells and endothelial cells both *in vitro* and *in vivo* [13, 14].

In this study, we investigated, by immunohistochemistry and RT-PCR, the expression of Orphanin, Oxytocin, Atrial Natriuretic Peptide, endothelial Nitric Oxide Synthase and inducible Nitric Oxide Synthase in full-term human umbilical cord.

Orphanin FQ is a peptide mainly involved in the mediation and modulation of pain; intracerebroventricular injection of Orphanin into a mouse brain has hyperalgesic effects [15, 16]. Orphanin has been shown to be involved in anxiety, stress, memory, learning, locomotor activity, tolerance, dependence and withdrawal phenomena and it also has a role in vasorelaxation; it causes hypotension and vasodilatation.

Oxytocin is synthesized in magnocellular neurosecretory cells of supraoptic and paraventricular nuclei of the hypothalamus and is released by the pituitary gland. Based on its functions, it can be considered to be both a hormone and a neurotransmitter; as a hormone, it stimulates uterine contractions during labor and milk production in breastfeeding, and, due to its similarity to vasopressin, can reduce the excretion of urine and stimulate sodium excretion. As a neurotransmitter, Oxytocin has a role in social behavior in many species; it is involved in bonding, maternal behavior, sexual arousal, increasing trust and reducing fear [17, 18]; it also has anti-stress functions: for example it reduces blood pressure and cortisol levels, increases tolerance to pain and reduces anxiety.

Atrial Natriuretic Peptide (ANP) is synthesized by atrial myocytes and released in response to high blood pressure, atrial distension, sympathetic stimulation of $\beta$-adrenoreceptors, high sodium concentration, angiotensin-II and endothelin. Its function is to reduce blood pressure through the reduction of water and sodium in the circulatory system, but it also has vascular effects: it relaxes vascular smooth muscles in arterioles and venules.

Nitric Oxide Synthase (NOS) is the enzyme responsible for the synthesis of nitric oxide (NO), a molecule involved in vasorelaxation, neurotransmission and cytotoxicity. Three isoforms exist: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). eNOS is the constitutive isoform expressed mainly by endothelial cells; it provides a basal level of nitric oxide and has a pivotal role in vasorelaxation, platelet aggregation and the mechanisms of cardiovascular homeostasis. iNOS is the inducible isoform expressed by macrophages and is activated by inflammatory signals such as cytokines, interferon-$\gamma$ and oxidative stresses.

Umbilical cord blood vessels lack any innervations, thus endothelial cells must play a major role in the local control of blood flow [19]; this prompted us to investigate the expression of some peptides involved in the regulation of vascular tone: we studied the expression and localization of Orphanin FQ, Oxytocin, ANP, eNOS and iNOS in full-term human umbilical cords by immunohistochemistry and RT-PCR.

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## Material and methods

**Samples.** The analysis was performed on 15 full-term human umbilical cords obtained by cesarean section. The umbilical cords were provided by the Gynecology and Obstetrics clinic of the Paolo Giaccone University Hospital in Palermo. Human gingival mucosa samples used as positive controls were provided by the Odontostomatological clinic of the same hospital. Informed consent was obtained from patients a few days before surgery. The experiment was approved by the local Ethics Committee.

**Animals.** Rat tissue samples, used as positive controls for some of our immunohistochemical experiments, were obtained from male Wistar rats (four months old). Animals were housed three to a cage in plastic cages lined with wood chips, alternating 12 h light/dark periods (lights on 6 am–6 pm) in a temperature (24 ± 2°C) and humidity-controlled room. The rats had free access to water and food. Before being sacrificed, rats were anesthetized by intraperitoneal injection of chloralose. Animal experiments were performed in accordance with the European Communities Council Directive (86/609/EEC). All appropriate measures were taken to minimize pain or discomfort to the animals. Brains, hearts and aorta were dissected.

**Immunohistochemistry.** Tissue samples (human gingiva, rat tissues or human umbilical cord sections) were formalin-fixed, dehydrated in a series of alcohols, clarified in xylene and paraffin-embedded. Sections of 7 $\mu$m were cut with a Leica microtome RM2145, dried overnight at 37°C and then stored at room temperature until use; prior to immunostaining, slides were de-waxed in xylene, re-hydrated in a series of alcohols and rinsed in distilled water for five minutes.

Immunohistochemistry was performed using the DakoCytomation EnVision + System-HRP (AEC) kit from Dako, following the manufacturer's instructions. The sections were covered with peroxidase block reagent and incubated for five minutes at room temperature, then rinsed once in Phosphate Buffer Saline (PBS) pH 7.2, covered with the antibody solution and incubated at 4°C overnight. Rabbit Anti-Orphanin FQ Polyclonal Antibody (Chemicon) (1:500 dilution), Rabbit Anti-Oxytocin polyclonal antibody (Chemicon) (1:800 dilution), Rabbit Anti-ANP polyclonal antibody (Chemicon) (1:800 dilution), Mouse Anti-eNOS/NOS type III monoclonal antibody (BD Biosciences) (1:25 dilution) and Rabbit Anti-iNOS/NOS type II polyclonal antibody (BD Biosciences) (1:25 dilution) were used. As negative controls for our antibodies, we used mouse IgG1 antibody (Dako, clone DAK-G01) (1:100 dilution) and rabbit immunoglobulin fraction (Dako) (1:200 dilution). All antibodies were diluted in a 0.1% bovine serum albumin (BSA) solution.

Rat brains were used as positive controls for Orphanin because of the well-documented expression of Orphanin in the pituitary gland; the Rabbit Anti-Orphanin FQ Polyclonal Antibody used was also able to recognize the rat protein. Rat brains were also used as positive controls for Oxytocin, since Oxytocin is expressed in the rat hypothalamus; the polyclonal antibody used in this study was able to recognize rat Oxytocin. Rat hearts were used as positive controls for ANP, since ANP is synthesized by atrial myocites; the polyclonal antibody used was able to recognize rat ANP. Rat aorta was used as positive control for eNOS, due to eNOS having endothelial expression; the monoclonal antibody used also recognized rat protein. Human gingival mucosa samples from patients with epulis were used as positive controls for iNOS since epulis is characterized by inflammation and presence of macrophages. Negative control sample was subjected to the same treatment, but the primary antibody was omitted.

Samples were rinsed twice in PBS pH 7.2, incubated with peroxidase-labeled polymer reagent for 30 minutes, rinsed twice in PBS pH 7.2 and incubated with substrate-chromogen reagent. The staining reaction was stopped by rinsing the slides in distilled water and the staining time was the same for all the samples. Slides were coverslipped using the DakoCytomation Faramount Aqueous Mounting Medium (Dako, Glostrup, Denmark). The specimens were observed under a Leica DM1000 light microscope.

**Semi-quantitative evaluation of immunoreactivity.** The intensity of immunostaining showed in Table 1 was evaluated by three independent observers according to the following criteria: (–) no staining, (+) light staining, (+ +) moderate staining, (+ + +) intense staining.

**Total RNA extraction.** Samples of human umbilical cord were frozen in liquid nitrogen immediately after cesarean

**Table 1.** Immunohistochemical results

| | Epithelium | Wharton's jelly | Vessel musculature | Endothelium |
|---|---|---|---|---|
| Orphanin | – | + + | + | + + + |
| Oxytocin | + | + + + | – | + + + |
| ANP | + + + | + + + | – | + + + |
| eNOS | + + | + + | – | + |
| iNOS | – | – | – | + + + |

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**Table 2.** Names, sequences and amplification product size of PCR primers used in this work

| Primer name | Primer sequence | Product size |
|---|---|---|
| Orphanin human forward | GTC CTG CTT TGT GAC CTG CT | 159 base pairs |
| Orphanin human reverse | GCT GGG GAA GAC CTT CTC TT | |
| Oxytocin human forward | CGC CTG CTA CAT CCA GAA CT | 193 base pairs |
| Oxytocin human reverse | CGG CAG GTA GTT CTC CTC CT | |
| ANP human forward | GCT GGA CCA TTT GGA AGA AA | 213 base pairs |
| ANP human reverse | TTG CTT TTT AGG AGG GCA GA | |
| eNOS human forward | ACC CTC ACC GCT ACA ACA TC | 198 base pairs |
| eNOS human reverse | GCT CAT TCT CCA GGT GCT TC | |
| iNOS human forward | CCC AAG CTC TAC ACC TCC AA | 284 base pairs |
| iNOS human reverse | TCC AGG ATA CCT TGG ACC AG | |
| GAPDH human forward | GAG TCA ACG GAT TTG GTC GT | 238 base pairs |
| GAPDH human reverse | TTG ATT TTG GAG GGA TCT CG | |

section and stored at –80°C until use. Total RNA extraction was accomplished using the Illustra RNAspin Mini Kit (Amersham Biosciences, Milan) following the manufacturer's instructions; RNA yields were evaluated spectrophotometrically (A260/A280) and RNA aliquots were stored at –80°C until use.

**RT-PCR (Reverse Transcription-Polymerase Chain Reaction).** For reverse transcription reaction, 2 $\mu$g of total RNA was used; to avoid DNA contamination of RNA samples, DNAse digestion was performed using AMPD1 kit (SIGMA). RT reaction was performed using the Enhanced avian HS RT-PCR kit (SIGMA) following the manufacturer's instructions: 1 $\mu$l random nonamers and 1 $\mu$l anchored oligo (dT)23 were added to the DNAse digestion product and incubated at 70°C for ten minutes to denature the sample, then 2 $\mu$l 10 × Buffer, 1 $\mu$l deoxynucleotide mix, 1 $\mu$l RNAse inhibitor, 1 $\mu$l Enhanced AMV Reverse Transcriptase enzyme and DEPC water were added to the sample. RT reaction was performed in 20 $\mu$l total volume at 42°C for 50 minutes, followed by a step at 95°C for five minutes to inactivate the enzyme.

PCR was performed using the PCR enzyme Selection Kit — high specificity (Invitrogen) following the manufacturer's instructions: 2 $\mu$l of template DNA, 0.5 $\mu$l of the primers mix (200 nM final concentration) and 22.5 $\mu$l of the Platinum Super Mix were mixed together. The components were denatured at 94°C for three minutes, then 40 cycles of 94°C 60 seconds, 56°C 60 seconds, 72°C 60 seconds, with a final extension step at 72°C for ten minutes. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as internal positive control and PCR products were visualized on 2% agarose gels stained with SYBR Green I nucleic acid gel stain (SIGMA); a fluorescent DNA intercalating agent. The sequences of the primers used in this work are shown in Table 2.

## Results

Orphanin FQ immunohistochemical expression was widely spread in full-term human umbilical cord, both in the stromal region and in the vascular compartment. The umbilical epithelium did not show any reactivity for Orphanin antibody. On the contrary, Orphanin reactivity was evident in Wharton's jelly mesenchymal cells, being equally distributed in all the thickness of the jelly, from the sub-epithelial region to the blood vessel musculature. An intense Orphanin protein expression was detected in the endothelial cells of all three umbilical vessels (Figure 2A), with intensity of immunoreaction and staining features seemingly identical in arteries and veins; in both cases brownish granules that filled the cytoplasm and masked the nucleus could be identified. Groups of muscle cells in vessel smooth musculature showed a clear reactivity for Orphanin antibody, particularly in the longitudinal muscle layer.

Oxytocin reactivity was moderate in the epithelial region with only part of the cells expressing the peptide. In these positive cells, the distribution of staining granules in the cytoplasm contrasted the nuclear negativity. Mesenchymal cells and fibroblasts in Wharton's jelly showed an intense immunoreactivity, with typical brownish granules occupying the entire cytoplasm of the cells (Figure 2B). Vessel musculature was negative for Oxytocin while endothelial cells were highly reactive (Figure 2C).

ANP protein expression was evident in the umbilical epithelium where many dark brown staining granules could be distinguished in the cytoplasm of the epithelial cells. Wharton's jelly cells showed an intense reactivity in all the jelly tissue, from the sub-

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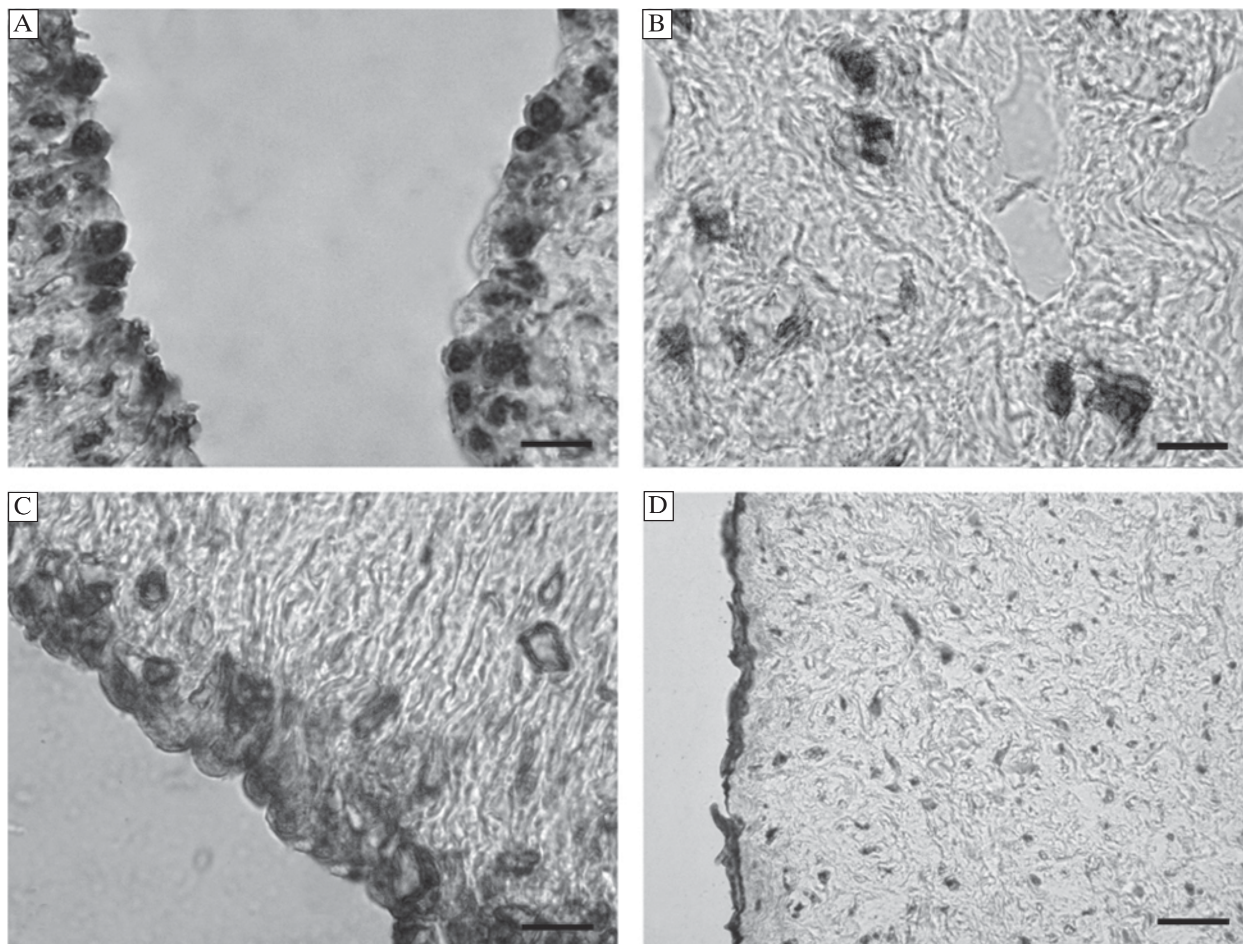


**Figure 2.** Immunohistochemistry in full-term human umbilical cord. **A.** Orphanin FQ in vascular endothelium (magnification × 100, bar 100 $\mu$); **B.** Oxytocin in Wharton's jelly cells (× 100, bar 100 $\mu$); **C.** Oxytocin in vascular endothelium (× 100, bar 100 $\mu$); **D.** ANP in the umbilical epithelium and in Wharton's jelly (× 20, bar 500 $\mu$)

epithelial region to the vascular compartment (Figure 2D). An intense ANP reactivity was identified in the arteries and vein endothelial cells (Figure 3A), while the smooth muscle layers around vessels were negative.

eNOS reactivity was evident in the umbilical epithelium and in Wharton's jelly cells, while being only moderate in the endothelium (Figure 3B). In this case smooth musculature around vessels also resulted negative. The distribution of positive cells in Wharton's jelly did not appear homogenous; the amount of fibroblasts expressing eNOS enzyme being higher in the region immediately below the umbilical epithelium, and decreasing in the inner jelly towards the vessels (Figure 3C).

iNOS protein expression was detected only in the endothelium of umbilical vessels; in endothelial cells of both vein and arteries, the staining granules filled the entire cytoplasm and the nuclei could not be distinguished (Figure 3D). The remaining regions of umbilical cord did not express iNOS.

Table 1 summarizes the immunohistochemical results for all the five proteins investigated.

Transcriptional expression of the genes encoding for these proteins was investigated through RT-PCR. This revealed that Oxytocin, ANP and eNOS mRNAs (Figure 4) were expressed in human umbilical cord, confirming the immunohistochemical data, while Orphanin and iNOS mRNAs were not detected.

## Discussion

Orphanin FQ expression in human umbilical cord is supported only by immunohistochemical data, while RT-PCR did not detect mRNA expression for this peptide. From this data, we could hypothesize that the protein we identify through immunoreaction is synthesized in another region of the body and delivered to the umbilical cord through the mother's blood flow. Interestingly, we found Orphanin protein expression mainly in the vascular compartment, in the endothelium and in the longitudinal musculature of

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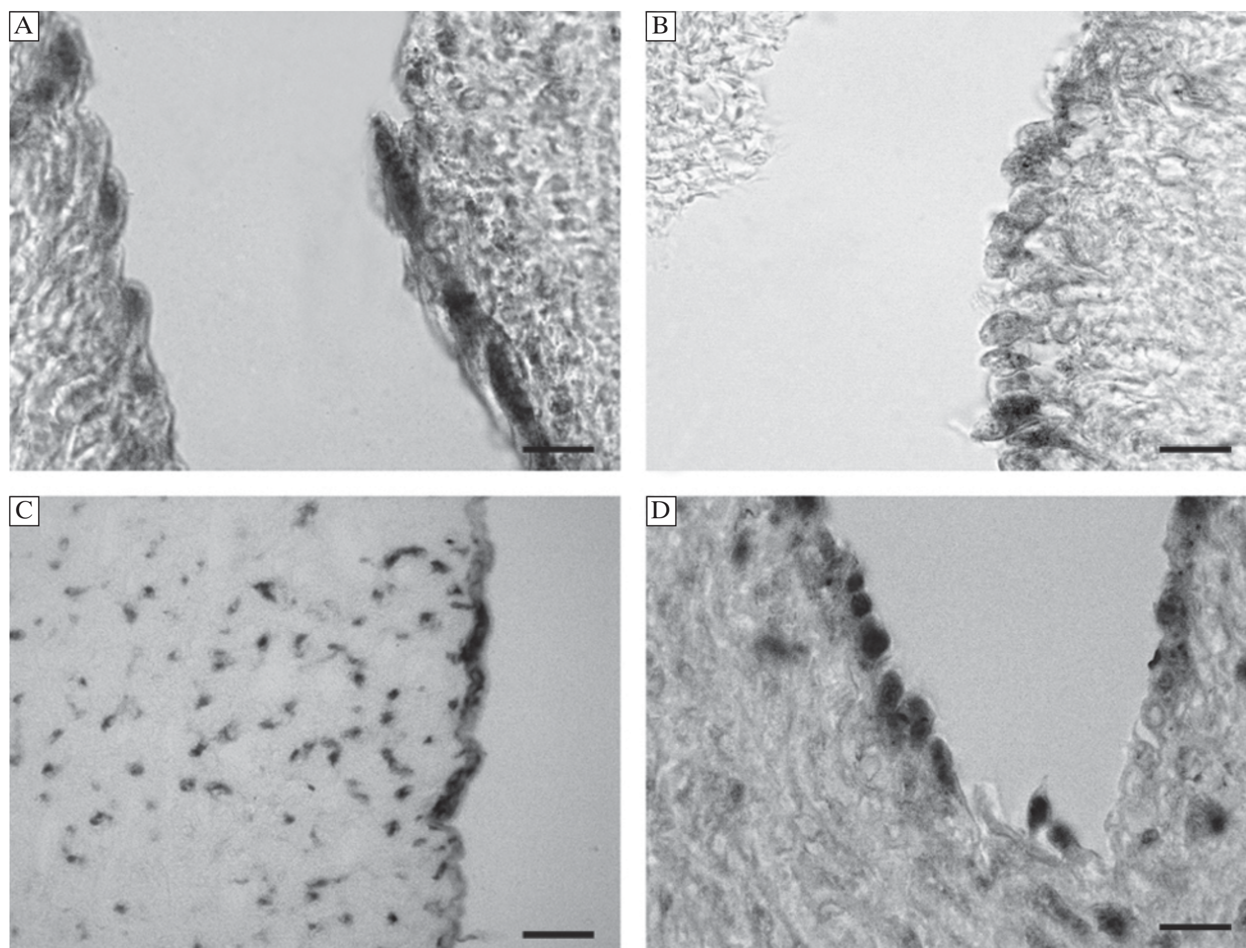


**Figure 3.** Immunohistochemistry in full-term human umbilical cord: **A.** ANP in vascular endothelium (× 100, bar 100 $\mu$); **B.** eNOS in vascular endothelium (× 100, bar 100 $\mu$); **C.** eNOS in the amniotic epithelium of the umbilical cord and in some cells of the sub-amniotic zone (× 20, bar 500 $\mu$); **D.** iNOS in vascular endothelium (× 100, bar 100 $\mu$)

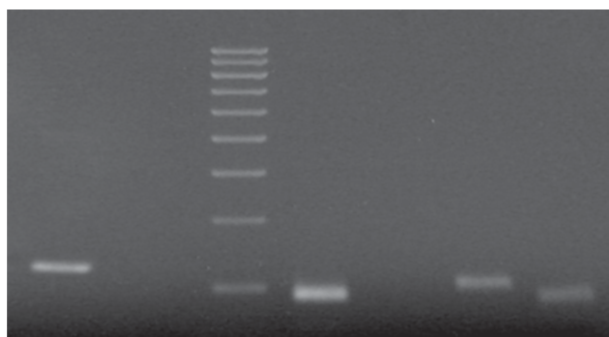

**Figure 4.** RT-PCR analysis in full-term human umbilical cord. Lane 1: GAPDH amplification product (238 base pairs); lane 2: no amplification product for Orphanin FQ; lane 3: 100 bp DNA ladder, from 1,000 base pairs to 200; lane 4: Oxytocin amplification product (193 base pairs); lane 5: no amplification product for iNOS; lane 6: ANP amplification product (213 base pairs); lane 7: eNOS amplification product (198 base pairs)

the vessels. In the literature, there have been many studies concerning the role of Orphanin in vasorelaxation: proinflammatory and vasodilatation effects have been reported in the rat mesenteric microcirculation and Orphanin relaxed the porcine coronary arterial rings, as well as inhibiting PGF-2$\alpha$-induced vasocontractility [20–22]. The Orphanin expression we identified in the vascular compartment of the umbilical cord suggests a role for this peptide in the regulation of vascular tone in this organ.

Oxytocin protein expression in the umbilical cord was mainly localized in the mesenchymal cells of Wharton's jelly and in the vessel endothelium; the immunohistochemical data was also confirmed by RT-PCR analysis showing transcriptional expression of the Oxytocin gene. In previous studies reported in the literature, Oxytocin has been shown to play an important role in the regulation of the vascular compartment: it causes vasodilatation in the pre-constricted pulmonary vasculature [23] and induces renal vasoconstriction in rats by activating vasopressin V1A receptors [24]. Moreover, pharmacological studies have shown the existence of Oxytocin receptors in human umbilical vein endothelial cells (HUVEC); the interaction between Oxytocin and its receptor causes

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an increase in the intracellular levels of calcium, activating the pathway responsible for nitric oxide production, thus leading to vasodilatation [25]. We hypothesize that Oxytocin exerts its role on these cells through an autocrine mechanism: the peptide is synthesized by HUVEC and acts on cell membrane Oxytocin receptors, starting the cascade of events that lead to NO production, release and finally to vasodilatation.

Oxytocin is typically synthesized by the hypothalamus, but some authors have demonstrated that it can also be produced by other tissues, for example the prostate gland [26]. The Oxytocin protein and gene expression we detected in the endothelium of umbilical cord vessels is in agreement with these results, and suggests that this peptide may also be synthesized by endothelial cells.

Atrial Natriuretic Peptide (ANP) immunoreactivity was localized mainly in the vessel endothelium and in Wharton's jelly cells. In spite of the well-known role of ANP in the regulation of muscle contractility, we did not detect ANP expression in the vascular musculature. RT-PCR confirmed ANP gene expression in the umbilical cord, suggesting that this peptide is synthesized by umbilical cord cells. Previous studies have demonstrated the expression of ANP in HUVEC [27, 28], where it has anti-inflammatory effects, reduces tumor necrosis factor-alpha (TNF-alpha)-induced activation and induces HUVEC survival in vitro [29, 30]. Other studies reported the co-localization of ANP with other vasoactive substances such as neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), substance P, calcitonin gene-related peptide (CGRP) and vasopressin [28] in umbilical vessels endothelium, suggesting ANP could be involved in a general mechanism of vascular homeostasis. Templeton et al. showed that physiological levels of ANP significantly reduced the vasoconstrictor effects of 5-hydroxytryptamine and endothelin-1 in the human umbilical artery. This suggests that ANP may modify vascular tone *in vivo*, thereby counterbalancing several humoral factors which act to increase vascular resistance within the fetoplacental circulation [31]. The expression of ANP in umbilical vessels endothelium we detected could contribute to vasodilator/vasoconstriction effects, and thus to the regulation of vascular tone.

Surprisingly, immunoreactivity for endothelial Nitric Oxide Synthase (eNOS) was moderate in the endothelium, while its expression resulted more evidently in Wharton's jelly mesenchymal cells. We also detected eNOS gene transcriptional expression through RT-PCR analysis, suggesting that eNOS is synthesized by umbilical cord cells. In agreement with our data, eNOS expression, typical of endothelia, has also been reported in other types of tissues and cells, such as many epithelium, fibroblasts, cardiomyocytes, osteocytes and glial cells [32–35].

iNOS expression in the human umbilical cord was detected only through immunohistochemistry, while RT-PCR analysis gave a negative result. We think that this discrepancy could mean that iNOS is synthesized in another region and then delivered to the umbilical cord through the mother's blood flow. iNOS protein expression was mainly localized in the endothelium; the nitric oxide synthesized by iNOS in the endothelium of umbilical vessels could contribute to vasodilatation and to the regulation of vascular homeostasis. In agreement with our findings, the expression of iNOS in HUVEC cells has been reported by many authors [36].

In conclusion, our study identified through immunohistochemistry the presence of Orphanin, Oxytocin, ANP, eNOS and iNOS proteins in full-term human umbilical cord, where stromal and endothelial compartments seem to be the main sources of these vasoactive peptides. Moreover, RT-PCR showed transcriptional expression of the genes encoding for Oxytocin, ANP and eNOS, suggesting that these molecules are synthesized by umbilical cord cells themselves, while Orphanin and iNOS are probably synthesized in other regions and delivered to the umbilical cord via the circulation.

The expression of these five vasoactive molecules in the human umbilical cord could be part of a general mechanism, realized mainly by endothelial cells, aimed at the local regulation of vascular tone, function and homeostasis of umbilical vessels.

It is already known that during trophoblast invasion into the basal decidua, an increase of diastolic flow occurs into the umbilical arteries. In this and in the following phases of gestation, it has been demonstrated that there is an increase in the expression of vasoactive peptides. Bloodstream evaluation in the uterus-placenta and fetus-placenta circulation (fluximetry), routine today in gestational clinical practise, shows the importance that vasoactive peptides can have on the homeostasis of the blood flow and thus on the health of the fetus [37].

The importance of vasoactive peptides in gestation is also highlighted by several fetal pathologies related to the phenomena of hypoxia and asphyxia, and by the intrauterine growth restriction syndrome (IUGR) that is often associated with an increase of resistance in one of the umbilical arteries.

Knowledge of the expression and distribution of these vasoactive peptides in the umbilical cord may help in understanding the histopathology of the feto-placental system and could have important clinical applications in the management of various syndromes that can develop during pregnancy or delivery.

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