Seminal plasma proteins and fertility indexes in the bull: The case for osteopontin

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Abstract

The ability to select high fertility sires results in the production of semen samples with optimal quality that will, ultimately improve conception rates. Currently, routine semen analysis based on motility and morphology provide useful, but limited information about fertility indexes in the male. Proven, high-use bulls from artificial insemination centers still show differences in non-return rates by as much as 20 to 25 %. The existence of subfertile sires with apparent normal semen quality is an important observation and has stimulated the search for other markers of fertility. Killian *et al.* (1993) reported the presence of four "fertility-associated proteins" in the Holstein seminal plasma, one of them later identified as osteopontin (OPN). The same protein, detected in accessory sex gland fluid, was associated with fertility indexes of dairy bulls. Osteopontin was first described in mineralized bone tissues as a cell adhesion component and its functions include cell migration, chemotaxis, calcification, tumor growth, and macrophage activation. This ubiquitous protein is expressed in the ampullae, seminal vesicles and possibly the epididymis, as well as in the oviduct. In the seminiferous tubules, where it is synthesized by Sertoli and germ cells, OPN is potentially involved in cell adhesion and migration. This review, therefore, summarizes key aspects of the osteopontin structure and expression in the male, and supports the notion that OPN plays a role in sperm-egg binding and fertilization.

Keywords: bulls, fertility, osteopontin, seminal plasma, sperm, testis.

Introduction: osteopontin and fertility indexes of dairy bulls

For the livestock and artificial insemination industry, production-tested sires with high fertilizing capacity are essential to assure optimal reproductive efficiency. Therefore, the search for indicators of male fertility has been the focus of several studies conducted in the past decades. Evaluation of sperm concentration and motility is frequently used to assess semen quality, but provides limited information about the potential fertility of sires (Elliot, 1978; Correa *et al.*, 1997; Rodriguez-Martinez and Larsson, 1998; Zhang *et al.*, 1998; Brahmkshtri *et al.*, 1999). Other criteria such as computerized analysis of motility and acrosome integrity also help to estimate semen quality and have been related to non-return rates of bulls, but correlations are not high or even consistent (Budworth *et al.*, 1982; Kjaestad *et al.*, 1993; Farrell *et al.*, 1998; Januskauskas *et al.*, 2000a,b). There is obvious evidence that motility is important for fertilization (Mortimer, 1997) but, within groups of bulls that have met a certain threshold for sperm motility, this parameter has only a limited contribution to detect differences in fertility scores (Flowers, 1997). Bulls from artificial insemination centers still show differences in non-return rates by as much as 20 to 25 %, but these results are not explained by routine semen analysis (Killian *et al.*, 1993; Larson and Miller, 2000). Thus, the existence of subfertile sires that appear to show normal semen quality is an important observation and has stimulated the study of other markers of fertility, such as molecular components of the seminal plasma (for review see, Braudmeyer and Miller, 2001).

The potential influence of seminal proteins on male reproduction came to attention because of the studies showing that their expression is associated with breeding scores of dairy bulls (Killian *et al.*, 1993; Cancel *et al.*, 1997), beef bulls (Bellin *et al.*, 1994, 1996; Parent *et al.*, 1999) and horses (Brandon *et al.*, 1999). Despite the relevance of those proteins, few have been identified (Cancel *et al.*, 1997; Gerena *et al.*, 1998; McCauley *et al.*, 1999, 2001) and in most cases our understanding of their function and relationships with fertility indexes is incomplete.

Killian *et al.* (1993) reported the presence of four "fertility-associated proteins" in the Holstein seminal plasma, two of which were later identified as prostaglandin D synthase (Gerena *et al.*, 1998) and osteopontin (Cancel *et al.*, 1997). More detailed statistical analysis confirmed that the density of osteopontin in 2D protein maps was responsible for 48 % of the variation in non-return rates of the bulls. Those fertility polypeptides were screened from electrophorectic maps of seminal plasma proteins and fertility indexes of bulls were based on return-rates calculated from at least 1,000 services/bull. This data set is unique because the large number of observations associated with each animal validates the fertility relationships found with the seminal plasma proteins(Amman and Hammerstedt, 2002). Recently, osteopontin was identified as one of the fertility proteins expressed in the accessory sex gland fluid and associated with dairy bull fertility (Moura *et al.*, 2005), in support of the original data published by Killian *et al*

(1993). Osteopontin was also identified by Western blots as one of the proteins related to field fertility scores of horses (Brandon *et al.*, 1999).

In general, osteopontin attributes are associated with cell adhesion, tissue remodeling, immune cell stimulation and chemotaxis, cell survival, inhibition of calcium oxalate crystallization, intra-cellular signaling, cytoskeleton dynamics and tumor metastasis (for reviews see: Denhardt *et al.*, 1995; Hoyer *et al.*, 1995; Liaw *et al.*, 1998; Denhardt *et al.*, 2001; Lessey, 2002; Mazzali *et al.*, 2002; Denhardt, 2004; Wai and Kuo, 2004). It is interesting that such a multifunctional protein is present in the male reproductive tract and related to fertility scores based on NRR of proven, high use dairy bulls. This review, therefore, summarizes key aspects of osteopontin structure and function, expression in the male reproductive tract and evidence indicating that OPN may be linked to key events of fertilization.

Structure and functional attributes of osteopontin

Osteopontin is an acidic protein, rich in aspartic acid, glutamic acid and serine (Sorenson and Petersen, 1994) and was initially isolated from the mineralized matrix of bovine bones (Franzen and Heinegard, 1985). Amino acid sequences from rat, mouse, human, pig and bovine osteopontin show 40 % homology, and the aspartic acid, glutamic acid and serine residues are those with the highest degree of conservation among those species. OPN belongs to a family of proteins named SIBLINGS (small integrin-binding ligand, N-linked glycoprotein), including dentin sialophosphoprotein, dentin matrix protein 1 and matrix extracellular phosphoglycoprotein (Denhardt, 2004). These proteins have similarities in DNA structure and gene location (they all appear in chromosome 5 in humans), expression in bone and mineralized tissues and the ability to activate matrix metalloproteinases through integrin receptors (Philip and Kundo, 2002).

Several conserved domains are present in osteopontin. The RGD sequence links two β sheets and is a site for integrin binding (Fig. 1). Antibodies against this RGD peptide blocks cell adhesion and its deletion also renders OPN incapable of activating integrin receptors (Butler, 1995). Sequences containing LPV bind $\alpha_4\beta_1$ and a domain formed by SVVYGLR binds $\alpha_9\beta_1$ after human OPN is cleaved by thrombin between amino acids R168 and S169 (Denhardt *et al.*, 2001). Resulting peptides from thrombin cleavage are from 28 to 35 kDa and enhanced ability to induce cell adhesion and chemotaxis (Helluin *et al.*, 2000). In addition to the thrombin cleavage site between residues Arg and Ser, bovine milk OPN appears to show another one, located a few amino acids before the RGD sequence, from the N-terminal. Sørensen *et al.* (1995) claim the existence of a unique cleavage site at Lys204 and Ala205 in the cow,

which should be located close to the C-terminal of the molecule, given the size of the bovine OPN (278 amino acids). Cleavage at that specific point must generate two fragments of very different molecular weights, but what triggers that cleavage in the bovine and the biological importance of it remain to be addressed. Integrins are not the only target for OPN's effect on cell-cell interaction. Osteopontin also binds to CD44 membrane glycoproteins through a domain independent of the RGD sequence (Fig. 1). This interaction takes part in the events of cell-cell interaction and signaling, migration and anti-apoptosis (Weber *et al.*, 1996).

A calcium binding domain is present between amino acids D216 and S228 in the human OPN and this 22-amino acid fragment is lacking in the protein isolated from cow milk (Kerr *et al.*, 1991), with the physiological implication of such unique characteristic still unknown. In humans, OPN prevents calcium crystal formation in the kidney, although this function of OPN is being elucidated (Mazzalli *et al.*, 2002). Osteopontin has two putative heparin-binding domains, located close to the SVVYGLR sequence (an integrin binding site) and between residues D298 and I305 (human OPN), which in turn is also a motif involved in CD44 binding. Those heparin-binding domains are based on amino acid sequence (Denhardt *et al.*, 2001) but they may be of importance to male reproduction because some seminal plasma heparin-binding proteins (HBP) have been associated with fertility scores of bulls (Bellin *et al.*, 1994, 1996; McCauley *et al.*, 2001). It is well established that there are HBPs secreted by the accessory sex glands that bind to ejaculated sperm and mediate biochemical events related to capacitation (Miller *et al.*, 1990). It is an intriguing coincidence, therefore, that osteopontin relates to bull fertility and also has putative heparin-binding domains but a potential role of OPN in the process of capacitation, like other HPBs, is still speculative.

The reported molecular weights of secreted osteopontin vary from 25 to 80 kDa (Denhardt *et al.*, 2001; Johnson *et al.*, 2003; Wai and Kuo, 2004). Bovine OPN, with 278 amino acid residues, has a predicted molecular weight of 41 kDa (Kerr *et al.*, 1991) but isoforms of 14 to 55 kDa have been found in seminal plasma and accessory sex glands (Cancel *et al.*, 1997, 1999). RNA splicing and a wide range of post-translational modifications, including cleavages, phosphorylation and glycosylation, account for the different OPN molecular weights detected in electrophoresis systems. The human and mouse genes have 7 exons, which consist of a source for regulatory post-transcription mechanisms (Yamamoto et al., 1995) and Sørensen et al. (1995) reported the existence of 28 phosphorylation and 3 O-glycosylation sites in the bovine milk OPN. These modifications occur in clusters of two and three amino acid residues in the protein and are thought to be important for OPN to regulate mineralization, calcification and macrophage-mediated responses

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in blood vessels. Although different isoforms of OPN have been found in the male reproductive tract, the biological significance of OPN post-transcription alterations has yet to be determined in this case.

H₂N

Transglutaminase binding residues: mediate OPN-OPN crosslinking and formation of polymers (Butler, 1995; Kaartinen *et al.*, 1999)

Hydroxyaspartite domain

Additional α4β1 integrin binding site

Binds to αvβ1,3,5,6; α5β1; α8β1 integrins

SVVYG: binds α9β1 and α4β1 integrins

Integrin-binding RGD domain connects two β sheets. One of these sheets is formed by the SVVY-GLRS motif

Metalloproteinase cleavage site

Thrombin cleavage site (replaced by K and S in the bovine)

Metalloproteinase cleavage sites: residues 201 and 210 (bovine OPN)

Additional thrombin cleavage site is present in the bovine OPN: Lys204-Ala205

Calcium-binding domain: D216 – S228 (human OPN)

This 22 aa fragment is absent in bovine OPN

Heparin-binding domain: D298 – I305 (human OPN); also binds to CD44 membrane receptors

305 aa

COOH

Figure 1. Diagram of human osteopontin structure. Based on information from Johnson *et al.* (2003); Wai and Kuo (2004); Denhardt *et al.* (2001); Butler (1995); Kerr *et al.* (1991).

Osteopontin in the male reproductive tract and evidence for its function

Osteopontin expression in the seminiferous tubules

Osteopontin has been immunolocalized in the basal and adluminal compartments of the rat seminiferous tubules and it is probably synthesized by the Sertoli cells (Siiteri *et al.*, 1995). In that same species, there is a 60-kDa protein reacting to OPN antibodies in spermatogonia and early spermatocytes (Luedtke *et al.*, 2002), a result in agreement with those published by Rodriguez *et al.* (2000), who detected mRNA in developing germ cells in the bull seminiferous tubules. OPN gene transcripts were associated only with certain stages of the seminiferous cycle (Rodriguez *et al.*, 2000), suggesting that its synthesis is also regulated by the major events that control spermatogenesis and germ cell de-

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velopment, as it happens to several proteins synthesized during spermatogenesis. One interpretation for the presence of OPN in the seminiferous tubules is that OPN functions as an anchoring protein between germ cells, Sertoli cells and components of the extracellular matrix. Osteopontin interacts with cell surface integrins, CD44, fibronectin and collagen (Murherjee *et al.*, 1995) and several integrin types are present in the membranes of peritubular cells, Sertoli cells and basement membranes of the tubules, acrosome of spermatids (Salanova *et al.*, 1995; Giebel *et al.*, 1997) and spermatogonial stem cells (Shinohara *et al.*, 1999). Integrins are important for cell migration through the compartments of the seminiferous tubules (Siu *et al.*, 2003) and may also mediate intracellular mechanisms in both germ and Sertoli cells through binding to ligands such as OPN. This binding induces intracellular signaling, through the NF-κB (Scatena *et al.*, 1998) and phosphatidilinositol 3-kinase/protein kinase B pathways (Hruska *et al.*, 1995; Wai and Kuo, 2004). An OPN knockout mouse model has been constructed, but never studied in detail regarding testicular or sperm function. In experiments conducted with those models to evaluate tissue remodeling and tumors, animals were described as "fertile" because they mate and produce normal litters (Liaw *et al.*, 1998). However, the focus of those studies was not the evaluation of reproduction and relative fertility was never evaluated in detail or subjected to any statistical analysis based on a reasonable number of experimental units.

Osteopontin expression as related to sperm function and fertilization

In bulls, osteopontin is secreted by ampullae and vesicular glands, indicating that the protein found in seminal plasma originates mainly from these glands. Within the epididymis, neither the protein nor its mRNA was detected in epithelial cells of the caput, corpus or cauda, although both of them were present on sperm within the lumen (Rodriguez *et al.*, 2000). In rats, OPN mRNA was found in the epididymal epithelium (Siiteri *et al.*, 1995) and immmunocytochemistry identified the protein in the cytoplasm of epithelial cells. Luedtke *et al.* (2002) also detected a 60-kDa protein in the rat epididymis. Although species may have different patterns of OPN expression in the epididymal cells and spermatozoa, it is possible that the contradictory results in the bull for OPN and its mRNA expression relate to differences in procedures and the amount of protein present in the tissues used for analysis.

The presence of osteopontin in the sperm is of major relevance for its potential role in the male. As stated above, OPN has been localized in both caput and cauda epididymal sperm, with staining on the dorsal part of head, midpiece and principal piece of the tail (Siiteri *et al.*, 1995). In bulls, more recent immunocytochemistry studies suggest similar patterns of OPN presence in ejaculated sperm (Erikson and Killian, 2004), with more intense staining in the portion of the head and midpiece. Western blots also reported positive reaction

of an anti-36 kDa bovine milk OPN antibody with membrane protein extracts from ejaculated sperm (Erickson *et al.*, 2003). Although these are preliminary results, it seems reasonable to state that OPN may be associated with both epididymal and ejaculated sperm. Epididymal spermatozoa may acquire OPN secreted from epididymal epithelial cells, although there is still doubt about the expression of OPN in the bovine epididymal epithelial cells. The fact that OPN is associated with Sertoli and germ cells in the seminiferous tubules raises the possibility that the later are carried out of the tubules to the epididymis already bound to OPN, but such hypothesis has yet to be tested experimentally. For ejaculated sperm, additional OPN could be acquired from the accessory sex glands. Integrins (αv and α5) have been identified in the bovine sperm membrane and can bind to oviductal OPN (Erikson and Killian, 2004). Human spesrmatozoa have the same αv and α5 integrin chains and cases of subfertility are associated with low expression of $\alpha_6\beta_1$ integrin receptors (Fusi *et al.*, 1996; Reddy *et al.*, 2003). It is tempting to speculate that OPN is inserted into the sperm membrane but characteristics of its structure and function point toward OPN being a component of the ECM instead. In the human gallbladder, osteopontin is seen as attached to layers of filamentous glycocalyx on the surface of epithelial cells, in the Golgi apparatus and inside secretory cytoplasmic vesicles (Hong *et al.*, 1994) and a proposed model for OPN function in the endometrium pictures it as part of the the ECM and not as an integral membrane protein (Johnson *et al.*, 2003; Spencer *et al.*, 2004).

Based on the molecular characteristics of osteopontin, experimental evidence and earlier hypotheses of Darribère *et al.* (2000) and Gabler *et al.* (2003), a model for a role of OPN sperm-egg interaction is presented (Fig. 2). According to this model, seminal plasma OPN binds to ejaculated sperm through integrin receptors. This complex binds to oocyte integrins, supported by the fact that oocytes expresses several types of integrin receptors (D'Cruz, 1996). This is a proposed model for osteopontin, although it is recognized that other proteins may also participate in sperm-egg interaction (D'Cruz, 1996; Kaji and Kudo, 2004; Shur *et al.*, 2004). Sperm integrins may bind OPN in the oviductal milieu given that osteopontin is also present in the bovine oviduct fluid and synthesized by oviductal epithelial cells (Gabler *et al.*, 2003). Moreover, spermatozoa integrin-OPN complex has the potential to bind other OPN moleculaes because osteopontin is capable of forming bonds with another OPN with high affinity, mediated by transglutaminases (Kaartinen *et al.*, 1999). Cross linking with more than two OPN molecules can also occur, although with lower tension (Goldsmith *et al.*, 2002). As happens with the OPN-OPN matrix, interactions between integrins and RGD containing peptides are very strong and require a great deal of mechanical force to be pulled apart (Lehenkari and Horton, 1999; Li *et al.*, 2003). This interaction occurs through a shallow groove between α and β units of integrin and a

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protruding motif of RGD residues (Craig *et al.*, 2004), which links two β sheet domains in the case of OPN (Denhardt *et al.*, 2001). Surprisingly, this shallow tandem between integrin and an RGD-bearing ligand, like osteopontin, is considerably strong from one side but also allows fast attachment, making a suitable structural mechanism for participation in sperm-egg binding. Another possibility for OPN is through interaction with the CD44 transmembrane glycoprotein present in bovine sperm (Bains *et al.*, 2002) and oocyte (Schoenfelder and Einspanier, 2003). CD44 belongs to a family of hyaluronic acid binding proteins termed "hyaludherins", which have high affinity for hyaluronic acid and other components of the extracellular matrix such as collagens, osteopontin and metalloproteinases (Cichy and Puré, 2003). Moreover, CD44 mediates cell adhesion and signal transduction that leads to gene activation.

Besides the mere presence of OPN and integrins on sperm membrane extracts and integrins and CD44 on oocytes, evidence for the model shown in Fig. 2 is supported by a series of in vitro fertilization trials. Incubation of bovine oocytes with oviductal follicular fluid and antibodies against a 36-kDa bovine OPN significantly inhibited sperm binding to the zona pellucida and embryo development (Gonçalves *et al.*, 2004). Addition of purified 36-kDa milk OPN caused pronounced increases in cleavage rates at Day 4 and blastocyt development at Days 8 and 11 (Gonçalves *et al.*, 2003), suggesting that OPN participates in the event of sperm-egg binding, very likely forming a strong protein mesh to connect both male and female gametes in the oviduct. In a recent study focused on the OPN knockout model, embryo size was significantly reduced between gestational days 10.5 and 19.5 (Weintraub et al., 2004). These observations indicating that OPN affects the rate of embryo development in the early stages are in agreement with the results described above with bovine IVF trials.

The effect of OPN on events occurring after sperm-egg binding leaves little doubt that its interaction with oocytes may trigger intra-cellular signaling through second messenger pathways. Expression of osteopontin in both seminal plasma and oviductal fluid suggests a redundancy of functions alhough sperm leave the seminal fluid environment when semen is deposited in the female reproductive tract. Availability of OPN in the oviduct, where fertilization occurs, would ensure proper integrin and CD44 receptor saturation on the sperm surface, maximizing the chances for oocyte attachment. Given the alternatives for mRNA splicing, post-translational modifications and the versatility of OPN, it is possible that OPN secreted in the oviduct lumen affects the oocytes before they become in contact with the sperm.
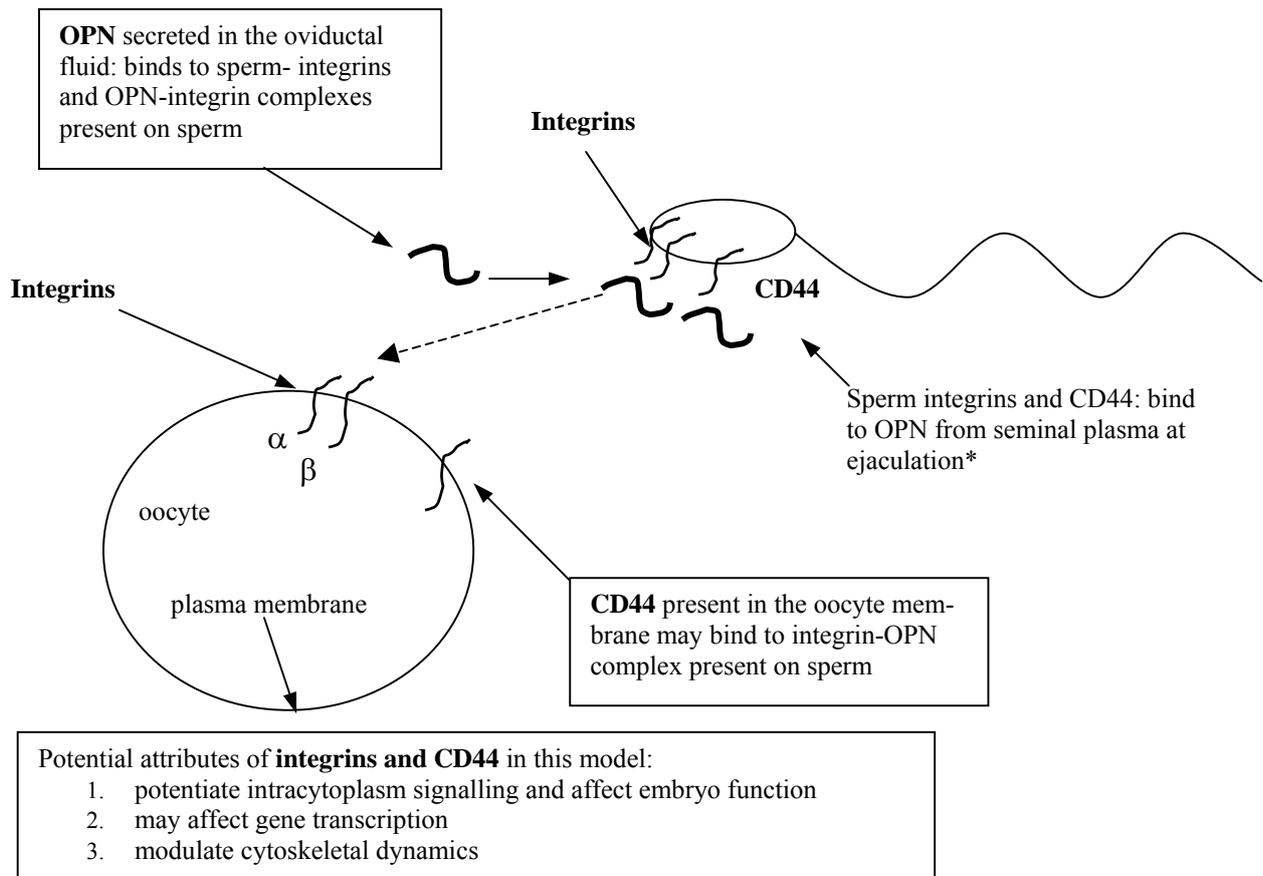
Figure 2. Suggested model for osteopontin interaction with ejaculated spermatozoa and oocytes. * As discussed in the text, additional OPN could bind the male gamete in the epididymis and even in the seminiferous tubules.

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Conclusions

In summary, we have presented the case of a multifunctional seminal plasma protein that has empirical relationships with fertility indexes of high performance sires used in artificial insemination centers. Starting from a given bull phenotype, osteopontin was identified as a "fertility protein" and subsequent studies unfolded part of its role in the male and fertilization. OPN precise function for Sertoli-germ cell interactions in the testis is conjectural, yet promising because of its unique attributes. Experimental evidence suggests that osteopontin affects sperm-oocyte binding and early embryo development, which is probably the reason why it was found associated with non-return rates of bulls. Given the complexity of germ cell existence and fertilization in mammals, it is concluded that osteopontin and also other proteins from the male reproductive fluids are potential markers of fertility. It is likely that, instead of the effect of a single component, the fertilizing potential of a given sire is influenced by interactions among several molecules present in the seminal plasma milieu. Establishing the identification of these components will help us to understand and diagnose cases of infertility and/or subfertility and enhance the accuracy for prediction of male reproductive performance.

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