Role of iron overload-induced macrophage apoptosis in the pathogenesis of peritoneal endometriosis

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Abstract

This article presents an overview of the involvement of iron overload-induced nitric oxide (NO) overproduction in apoptosis of peritoneal macrophages of women with endometriosis. We have postulated that the peritoneal iron overload originated from retrograde menstruation or bleeding lesions in the ectopic endometrium, which may contribute to the development of endometriosis by a wide range of mechanisms, including oxidative damage and chronic inflammation. Excessive NO production may also be associated with impaired clearance of endometrial cells by macrophages, which promotes cell growth in the peritoneal cavity. Therefore, further research of the mechanisms and consequences of macrophage apoptosis in endometriosis helps discover novel therapeutic strategies that are designed to prevent progression of endometriosis.

Introduction

Endometriosis is an estrogen-driven inflammatory disease that causes pelvic pain, painful menstrual periods, and infertility (Podgaec et al. 2007, Burney & Giudice 2012). It is characterized by the growth of endometrial tissue at extraterine sites, mainly on the ovaries and peritoneum. It affects ~10–15% of reproductive-aged women. However, the rates have risen by 30 and 50% in women undergoing laparoscopy to evaluate infertility or chronic pelvic pain respectively (Matarese et al. 2003, Becker & Amato 2007).

Despite extensive investigations, the pathophysiology of endometriosis remains elusive due to its multifactorial characteristics. Accumulated evidence indicates that genetic, hormonal, environmental, immunological, and anatomical factors either alone or in combination may determine the susceptibility to develop endometriosis (Defrere et al. 2006, Hull et al. 2008). The local environment is essentially important in the growth and progression of endometriotic lesions (Kobayashi et al. 2009). Severe hemolysis during retrograde menstruation, along with a defective or overwhelmed peritoneal disposal system in the case of increased menstrual reflux, results in iron overload in the peritoneal environment, which in turn permits attachment and growth of the endometrial cells or fragments (Gazvani & Templeton 2002). This iron accumulation might have numerous cytotoxic effects as it disrupts the balance between free radicals production and antioxidant defense, which leads to oxidative stress (OS) implicated in the pathogenesis of endometriosis. Several studies state that OS has adverse health effects on the pathogenesis of several diseases, including cancer, atherosclerosis, neurodegenerative disorders, and reproductive system diseases such as pre-eclampsia, and male and female infertility (Jackson et al. 2005, Rahman et al. 2012). Therefore, iron-induced OS may trigger the chain of events resulting in the development and progression of endometriosis (Van Langendonckt et al. 2002a, Carvalho et al. 2012).

In this article, we will summarize the current literatures focused on apoptotic cell death and nitric oxide (NO) with emphasis on the potential role of iron overload in these processes. Based on recent knowledge, we herein suggest that iron overload-induced macrophage apoptosis by overproduction of NO may play a significant role in the further establishment and growth of endometriotic lesions.

Iron-induced peritoneal OS in endometriosis development

Iron is an essential nutrient for many metabolic pathways of the body. Iron homeostasis is carefully regulated within the cells by various mechanisms. However, excess iron is considered as a threat to the cells and tissues. Iron toxicity is mainly related to its ability to catalyze the production of a wide variety of damaging...
free radical species in the Fenton reaction, leading to deregulation of cellular processes, cell dysfunction, and eventually to apoptosis or necrosis through lipid peroxidation, protein, and DNA damage (Papanikolaou & Pantopoulos 2005, Rahman et al. 2012).

The presence of iron overload has been demonstrated in various components of the peritoneal cavity of endometriosis patients (peritoneal fluid, macrophages, and endometriotic lesions; Defrere et al. 2008, Augoulea et al. 2012), which strongly suggests disruption of iron homeostasis in the peritoneal cavity of patients. Iron overload in the peritoneal fluid provokes oxidative injury and inflammatory response, involving peritoneal macrophages in particular, which promote the proliferative capacity of ectopic implants of endometrium in the peritoneal cavity (Van Langendonckt et al. 2002b, Szczeparska et al. 2003).

Generally, oxidative injury occurs when continued delivery of iron to the peritoneal macrophages is associated with inhibition of iron storage in ferritin (Hippeli & Elstner 1999). This iron accumulation in macrophages may severely compromise their functions. Excessive production of free radicals enhances the activity of nuclear factor kappa B (NFkB), which up-regulates the expression of multiple genes encoding pro-inflammatory cytokines, chemokines, adhesion molecules, growth factor, and angiogenic factor, supporting the inflammatory response, resistance to apoptosis, and cell proliferation in endometriotic lesions (González-Ramos et al. 2007, Loussu et al. 2009). The maintenance of high activity of p65 subunit of NFkB throughout the menstrual cycle owing to progesterone resistance has recently been demonstrated to stimulate an inflammatory response, which promotes the proliferation and survival of ectopic endometrial cell, thereby favoring the establishment and development of peritoneal endometriosis (González-Ramos et al. 2012). Macrophages also serve as the source of other inflammatory mediators contributing to the development of endometriosis, including NO. NO produced in abundance by the inducible form of NO synthase (iNOS, NOS2), induced by oxidant-sensitive transcription factors like NFkB, has the potential to exacerbate endometriosis by promoting inflammation and necrosis at the site of lesion (Beckman & Koppenol 1996, Detmers et al. 2000). Moreover, the peritoneal accumulation of pro-oxidant and pro-inflammatory factors during retrograde menstruation, such as hemoglobin and its by-product heme, may cause OS-mediated mesothelial layer damage and chronic inflammation, permitting the attachment and development of endometrial fragments in the peritoneum. Decreased expression of inducible HMOX1 (HO-1) by peritoneal macrophages and mesothelial cells impairs hemoglobin and heme detoxification in the case of endometriosis (Van Langendonckt et al. 2002c).

Altered immune responses play a prominent role in the development of peritoneal endometriosis due to failure to remove the ectopic endometrial cells (Giudice & Kao 2004, Mier-Cabrera et al. 2010). Numerous evidences indicate that the immune cells facilitate the establishment of endometriotic lesion and modulate endometriosis-associated pain and infertility as well (Berbic et al. 2009).

Macrophages are the predominant type of cell in the peritoneal fluid of patients with endometriosis. These cells are recruited in response to chronic stimuli such as ectopic endometrial implants as a foreign entity and/or excessive refluxed menstrual debris secreting high levels of chemotactic agents, including monocyte chemotactic protein 1 (MCP1), RANTES, and lyso-phosphatidylcholine (lyso-pc) (Oral et al. 1996, Santanman et al. 2002). Recruited macrophages are also highly active and release various mediators such as pro-inflammatory cytokines (IL1, IL6, and TNF2), prostaglandins, growth factors, and angiogenic factors and express inducible enzymes (iNOS and COX2) (Gazvani & Templeton 2002, Berkkanoglu & Arici 2003, Dmowski & Braun 2004, Dionyssopoulou et al. 2005). All these products mediate inflammation and neovascularization, thereby promoting the growth of implanted endometrial fragments (Lebovic et al. 2001, Gazvani & Templeton 2002, Matarese et al. 2003, Berbic et al. 2009). The differential expression of proteins involved in iron metabolism can affect the functional polarization of macrophages into classically activated M1 and alternatively activated M2 phenotypes (Cairo et al. 2011, Recalcati et al. 2012). The iron retention-prone M1 macrophages are characterized by up-regulated iron storage ferritin (FtH) that is accompanied by the down-regulation of transferrin receptor 1 (TIR1) and iron exporter ferroportin (Fpn) due to decreased activity of the iron regulatory protein 2 (IREB2 (IRP2)), a primary regulator of iron homeostasis within the cell, which in turn limits the labile iron pool (LIP) to protect themselves from oxidative damage (Coma et al. 2010, Recalcati et al. 2010). These cells perform several effector functions, including bacteriostatic activity, secretion of pro-inflammatory cytokines, immunostimulation, and tumor suppression, by inducing a polarized Th1 response (Gaetano et al. 2010, Recalcati et al. 2012). Hepcidin-mediated Fpn down-regulation has been reported to result in iron accumulation in M1 macrophages, which may contribute to wound healing or chronic inflammation (Recalcati et al. 2012). By contrast, immunosuppressive M2 macrophages resolve inflammation and promote parasite killing, angiogenesis, wound healing, matrix remodeling, and tumor growth by increasing the availability of extracellular iron and polarized Th2 responses (Brunelli & Rovere-Querini 2008, Coma et al. 2010, Gaetano et al. 2010, Recalcati et al. 2010). The phenotypic characteristics of these cells are FtHlow/Fpnhigh/TIR1high/hemoglobin–haptoglobin–scavenger receptor CD163high/hemopexin-bound heme.
receptor CD19\textsuperscript{high}/HMOX1\textsuperscript{high} with high capacity for iron release which may affect other cells in the microenvironment (Gaetano et al. 2010, Recalcati et al. 2012). Capobianco et al. (2011) demonstrated a supportive role of CD163\textsuperscript{+} pro-angiogenic Tek (Tie2)-expressing macrophages in angiogenesis and accelerated growth of ectopic lesions.

The inflammatory peritoneal environment characterizes macrophage polarization toward the M2 phenotype expressing markers of alternative activation, particularly high levels of scavenger receptors, CD163 and CD206, which are involved in the export of heme-derived iron and removal of inflammatory mediators respectively (Smith et al. 2012, Capobianco & Rovere-Querini 2013). Impaired ability of peritoneal macrophages to dispose apoptotic endometrial remnants and defective scavenging heme-bound iron, resulting from cyclic progesterone withdrawal, may activate macrophages recruited at sites of local hypoxia and tissue stress (Capobianco & Rovere-Querini 2013). The activation of peritoneal macrophages by the environmental cues such as local hypoxia and iron overload may provide a permissive environment to vascularization and growth of ectopic endometriosis lesions via reduced immune clearance of endometrial cells (Bacci et al. 2009). It should be noted that plasticity of macrophage polarization may occur in endometriosis. Tissue damaging as a consequence of sustained M1 polarization may be associated with an increase in the response of the M2 cells, which supports the persistent growth of ectopic endometrial tissue (Smith et al. 2012).

**Pleiotropic activities of NO in the endometrium**

An increase in the number and activity of macrophages is also accompanied by release of NO (Gupta et al. 2006). NO is a highly reactive multifunctional molecule with dichotomous regulatory roles in both physiological and pathological processes (Chen et al. 2001), which is synthesized enzymatically from l-arginine by three isoforms of NOSs, including neuronal NOS (NOS1 (nNOS)), inducible NOS (NOS2), and endothelial NOS (NOS3 (eNOS)) (Karpuzoglu & Ahmed 2006, Mori 2007). NO is well known to act as a neurotransmitter and vasorelaxant agent, and is involved in antimicrobial defense mechanisms (Guix et al. 2005). With respect to apoptosis, NO exerts both pro- and anti-apoptotic actions. The complexity is attributed to its interactions with other molecules such as iron, reactive oxygen species (ROS), metal ions, and proteins (Chung et al. 2001, Kim et al. 2001). Induction of NOS2, particularly in mononuclear phagocytes (monocytes and macrophages), produces excessive NO in response to numerous stimuli such as tissue injury, inflammation, cytokines, and growth factors (Rosselli 1997). This may be accompanied by increased ROS production, exacerbating tissue injury. NO reaction with superoxide anion generates a strong oxidant peroxynitrite (OONO\textsuperscript{-}) which can react with biological molecules in a number of mechanisms, particularly oxidizing iron/sulfur centers, zinc finger, and protein thiols (Beckman & Koppenol 1996, Detmers et al. 2000, Liu et al. 2005).

In the reproductive system, NO plays an important physiological role in female reproductive processes, including ovulation, sperm motility and fertilization, implantation, pregnancy, and labor (Ledingham et al. 2000, Wu et al. 2003). Excessive generation of NO by activated macrophages negatively affects fertility by changing the composition of the peritoneal environment and impairing ovulation, fertilization, and early embryonic development (Agarwal et al. 2005, Gupta et al. 2006).

NO, along with prostaglandin E\textsubscript{2} and prostacyclin I\textsubscript{2}, participates in the initiation and control of menstrual bleeding (Tellier et al. 1997). Luteal phase progesterone up-regulates NOS3-derived NO production from both glandular epithelium and endometrial vessels, leading to vasodilation and endometrial receptivity. After onset of bleeding as a consequence of progesterone withdrawal, an increased NOS2-generated NO, which is induced by pro-inflammatory cytokines produced by endometrium, including IL1 and TNF\alpha, may on one hand maintain vasodilation and on the other hand inhibit platelet aggregation (Cameron & Campbell 1998, Tschugguel et al. 1998, Chwalisz & Garfield 2000). Furthermore, NO contributes to metalloproteinase-mediated cyclical destruction of endometrium and induction of apoptosis in endometrial cells by reversing the balance between anti-apoptotic BCL2 and pro-apoptotic BAX proteins observed during menstruation or embryo implantation (Cameron & Campbell 1998, Chwalisz & Garfield 2000, Castro et al. 2002). Sex steroids are also involved in the regulation of apoptosis in human endometrium. Blocking the FAS/FASLG(FASL)-mediated apoptosis in the proliferative endometria via estrogen-mediated BCL2 expression is associated with the retention of co-expressed FAS and FASLG within the cells that is followed by BAX expression and the FAS/FASLG-mediated apoptosis of endometrial cells in response to progesterone withdrawal, either in an autocrine or paracrine manner by soluble FASLG (Song et al. 2002). Li et al. (2001) established that NO synergistic with progesterone may enhance apoptotic susceptibility of endometrial epithelial cells by increasing the activation of the p38 MAPK pathway, which facilitates trophoblast implantation and invasion. Progesterone may affect the up-regulation of NOS3 and NOS2 expression in endometrial epithelial cells and also stimulation of NOS3 phosphorylation through both the PI3/Akt and the ERK1/2 pathways (Khoram & Han 2009). The differential regulatory function of NO has also been confirmed by l-arginine treatment followed by a significant increase in apoptosis of proliferative not secretory endometrial implants. This indicates more sensitivity of the cells for activation of apoptotic
machinery that has not already been induced by high generation of local endogenous factors, such as cytokine and NO (Castro et al. 2002, Johnson et al. 2004). Therefore, the endometrial microenvironment during the proliferative phase has been suggested to affect the response of the secretory endometria in the menstruation process (Johnson et al. 2004).

The peritoneal fluid in women with endometriosis and/or unexplained infertility has been shown to contain high levels of NO (Lee et al. 2004). Induction of NOS2 and hence NO synthesis by macrophages have increased in response to IFNγ and TNFα with lipopolysaccharide (LPS) in the endometriotic peritoneal fluid (Alpay et al. 2006). The endometrium of women with endometriosis and adenomyosis exhibited persistent overexpression of NOS3 throughout the menstrual cycle, which was associated with chronic inflammation damaging early embryonic development (Ota et al. 1998, Cao et al. 2004). The up-regulation of glandular NOS3 in association with reduced expression of αβ1 integrin in response to high levels of local inflammatory cytokines and estrogen may enforce a positive feedback loop in the further growth of implanted lesions by activating cyclooxygenase-2 and aromatase (Khorram & Lessey 2002). Chen et al. (2001) demonstrated that embryo apoptosis induced by excessive amounts of NO is followed by embryo fragmentation and subsequently early embryo loss, implying that this may be a contributing factor in endometriosis-associated infertility through the up-regulation of TP53 (p53) and BAX expression in a cGMP-independent pathway. A number of studies have found higher activity of NOS2 and more NO production by peritoneal macrophages from infertile women with endometriosis after immunological stimulation, which is an important determinant of the inflammation in the peritoneum (Dong et al. 2001, Osborn et al. 2002, Wu et al. 2003). GnRH analogs have been proven as effective factors in the treatment of endometriosis and adenomyosis by regulating the secretion of estradiol, resulting in marked reduction in NO production and peroxynitrite formation (Kamada et al. 2000). Recently, Greene et al. (2013) showed the regulatory role of ω-arginine in NO-mediated enhancement of endometrial growth through increased BAD phosphorylation, which is accompanied by reduced apoptosis. Nevertheless, further studies are necessary to elucidate the significance of NO in the pathogenesis of endometriosis.

### Molecular mechanisms of apoptotic cell death induced by NO

Recent studies have indicated that NO modulates apoptosis, or programmed cell death, in several cell types, including inflammatory cells (Shaw et al. 2005). Apoptosis, characterized by a series of morphological and biochemical alterations, eliminates excess or unwanted cells from tissue without inducing an inflammatory reaction to maintain tissue homeostasis (Vignali et al. 2002). However, apoptotic deregulation is implicated in the pathogenesis of several human diseases (Nakagawa et al. 2000). A variety of extrinsic and intrinsic signals trigger apoptosis. NO has recently been recognized as an inducer of apoptosis (Brüne et al. 1998a); however, the effect of NO on cell apoptosis is controversial. On one side, NO provokes apoptosis, while on the other side, it protects cells from apoptotic cell death. This can be partly attributed to the rate of NO production, but to a large extent may be dependent on intracellular redox state and redox capacity within cells (Weigert & Brune 2008). NO-induced DNA damage has been demonstrated to result in p53 accumulation that activates caspase family proteases involved in both the mitochondrial and Fas/Fasl signaling pathways (Bahat et al. 2004). The endoplasmic reticulum (ER) stress-CHOP pathway may also account for p53-independent cell death when p53 response is not predominant (Brüne 2003). The mitochondria-dependent apoptosis pathway is initiated by the down-regulation of anti-apoptotic proteins, e.g., BCL2 and BCLXL (BCLXL), concurrent with the up-regulation of pro-apoptotic proteins, e.g., BAX, BAK1 (BAK), and BID (Li & Wogan 2005). Pro-apoptotic proteins act either by opening mitochondrial permeability transition pores directly resulting in release of some pro-apoptotic factors such as cytochrome c, AIF, endonuclease G, and smac/DIABLO (second mitochondrial derived activator of caspase/direct IAP-binding protein with low pi) or by binding to anti-apoptotic proteins to prevent their actions (Guha et al. 2006; Fig. 1). NO can result in inhibition of mitochondrial respiration, thereby leading to depletion of

![Figure 1: Schematic summary of alterations in apoptotic signaling pathways under NO treatment](https://www.reproduction-online.org)
ATP that stimulates superoxide anion (O$_2^-$) production by competing with molecular oxygen for binding sites on cytochrome $c$ oxidase in mitochondria and then its reaction with NO to form peroxynitrite (ONOO$^{-}$) (Oyama et al. 2002). Superoxide formation may also occur by other enzymatic routes, for example, xanthine oxidase induced by TNF-$
abla$ and IFN-$
abla$ (Hu & Brindle 2005). Overexpressed xanthine oxidase localized to glandular epithelium has been observed in adenomyosis and endometriosis during early secretory phase of menstrual cycle (Ota et al. 2001). Excessive generation of NO or peroxynitrite induces apoptosis through multiple mechanisms, including irreversible inhibition of complex I by s-nitrosation that is followed by inhibition of mitochondrial aconitase and complex III through removal of iron from Fe–S centers, which in turn results in mitochondrial dysfunction, cytochrome $c$ release, and consequently caspase-dependent apoptosis (Li & Wogan 2005). It has previously been reported that NO and peroxynitrite can react with Fe–S clusters of cytosolic aconitase, which induces the transition from aconitase to iron regulatory protein 1 (ACO1 (IRP1)) as a consequence of total cluster disruption and causes an increase in the LIP by repressing ferritin translation and reduction in ferritin pool (Bouton et al. 1997, Murgia et al. 2002, Galleano et al. 2004). Ferritin synthesis is considered as a part of cytoprotective responses to control oxidative damage in the setting of iron overload. Thus, ablation of the induction of ferritin synthesis has already been demonstrated to inhibit NO-induced apoptosis through PARP cleavage (Messer & Brüne 1995).

The apparent role of apoptosis in the physiopathology of endometriosis has gained a lot of interest. Accumulated evidence indicates that decreased apoptosis of endometrial cells in a phase-dependent manner during the menstrual cycle may facilitate ectopic survival of implanted endometrial fragments (Dmowski et al. 2001, Garcia-Velasco & Arici 2003). In healthy women, the endometrium that is refluxed into the peritoneum undergoes apoptosis called anoikis because of its failure to interact with the extracellular matrix required for the growth of ectopic endometrium (Béliard et al. 2004). The resistance of endometrial cells to apoptosis and their increased sensitivity to proliferation is one of the proposed theories for the development of endometriosis (Harada et al. 2004). Intrinsic resistance of refluxed endometrial cells to apoptosis may promote their adherence to the peritoneal mesothelial cells, thereby provoking cell proliferation and neoangiogenesis to develop active endometriosis (Park et al. 2009). Meresman et al. (2000) confirmed the pathological role of apoptosis in endometriosis by detecting up-regulated expression of the anti-apoptotic factor BCL2 that is accompanied by reduced expression of the pro-apoptotic factor BAX. Moreover, the location of endometriosis determines the expression level of pro-apoptotic proteins involved in the different apoptotic pathways (Dufournet et al. 2006).

In eutopic endometrium from women with endometriosis, it has been indicated that up-regulation of MYC (c-Myc) by estradiol in the absence of TGFB1 is associated with decreased BAX expression reducing apoptosis in the late secretory phase in these patients compared with normal endometrium, favoring survival and implantation of ectopic endometrial cells (Johnson et al. 2005).

The iron burden that is imposed on peritoneal macrophages by unrestrained phagocytosis of refluxed erythrocytes, together with overwhelmed HMOX1-dependent heme degradation, may be lethal to the cells and subsequently result in release of pro-oxidants such as iron and heme into the peritoneum (Defrere et al. 2008, Louise et al. 2012). Macrophages have specific sensitivity to NO-mediated apoptotic cell death in a cGMP-independent manner, which may be associated with inhibition of protective proteins such as heme oxygenase, metallothionin, and heat shock proteins (Brüne et al. 1998b, Kim et al. 1999, Choi et al. 2002). Long-term activation can induce apoptosis in peritoneal macrophages. However, an increase in the proportion of highly active BCL2-positive peritoneal macrophages during both phases of the menstrual cycle in women with endometriosis may have important effects on the growth and vascularization of ectopic endometrium (McLaren et al. 1997). Bcl2 overexpression has already been demonstrated to inhibit NO-induced apoptosis through PARP cleavage (Messmer & Brüne 1995).

The Fas/FasL death pathway is one of the major apoptotic pathways. Apoptosis of eutopic and ectopic endometrial tissues may be less relied on FAS expression (Agic et al. 2009). A study by Garcia-Velasco et al. (2002) showed that elevated apoptosis of FAS-expressing immune cells in the peritoneal cavity of women with advanced endometriosis is attributed to high levels of soluble FASLG, impairing the removal of ectopic endometrial cells. This suggests a role for apoptotic deregulation in the pathophysiology of endometriosis. Elevated macrophage-derived growth factors in the peritoneal fluid up-regulate the expression of FASLG involved in apoptosis of activated T cells and macrophages within the ectopic endometrial implants. This leads to the development of immune tolerance and long-term survival of endometrial cells by increasing resistance to macrophage-mediated cytolysis during the initial attachment in the peritoneal cavity (Ota et al. 1998, Garcia-Velasco et al. 1999, Garcia-Velasco & Arici 2003, Agic et al. 2009). Accordingly, iron overload in peritoneal macrophages of women with endometriosis may play a pathological role in the development and progression of the disease.
Conclusion
Although mitochondria (intrinsic pathway) and/or Fas death receptor (extrinsic pathway) contribute to macrophage apoptosis, the cell death cascades need to be fully clarified during NO-mediated apoptosis. Based on the reviewed studies, iron overload-induced OS may permit ectopic survival of endometrial cells and lesion vascularization in the peritoneal cavity. It is tempting to propose that excessive NO in the peritoneal fluid of women with endometriosis may play a role in the iron overload-induced mitochondrial and/or death receptor apoptosis pathways to cause progression of endometriosis. NO and ROS have recently been demonstrated to disturb ER functions as ER stressors, which in turn trigger apoptosis (Gotto & Masataka 2006). NO overproduction has the potential of ER stress-induced apoptosis implicated in the pathogenesis of various human diseases, including atherosclerosis, diabetes, heart failure, and neurodegenerative disorders (Okada et al. 2004, Liu et al. 2005, Xu et al. 2005, Malhotra & Kaufman 2007, Tabas 2010). Therefore, further studies must aim to elucidate the exact role of apoptosis pathways through the mitochondrial and/or ER stress pathways in the disease, and determine whether modulators of NO synthesis and ER stress with iron chelators can provide a useful basis for new therapeutic approaches.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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