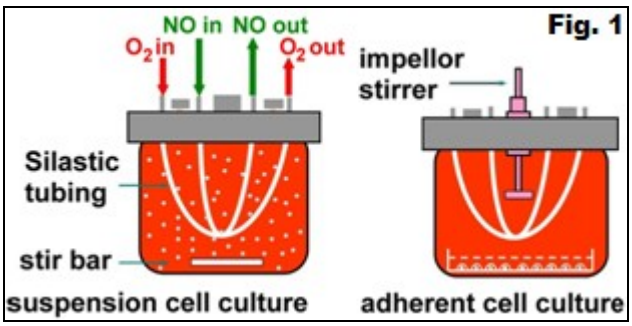
**Title:** Improvement of a Novel Gas Delivery System for Cell Culture Studies and Investigation of the Effect of Nitric Oxide on Cells Susceptible to Cancer-Causing Inflammation

**Keywords:** nitric oxide, chronic inflammation, DNA lesions, p53, apoptosis, reactive oxygen species, reactive nitrogen species, peroxynitrite, superoxide, nitrosoperoxycarbonate

**Background:** There is an increasingly large body of epidemiological evidence linking chronic inflammation to a variety of different cancers, including those of the gastrointestinal tract, liver, and lungs. The detailed mechanism of this link is not well understood, and the search for it is one of the most important endeavors in modern cancer research. The most promising possibility for this link is long-term exposure to reactive species produced by macrophages and neutrophils at sites of inflammation[1]. Researchers at the Massachusetts Institute of Technology have identified nitric oxide as an essential mediator in the development of many of these cancers.

Nitric oxide (NO) is both essential for function (as a component in cell signaling and in the non-specific immune response) and potentially destructive (as a mutagen and cytotoxin)[2]. Attempts to define quantitatively its *in vivo* effects have been hampered by numerous methodological problems. Techniques for delivering NO to cultured cells have included NO donor compounds, addition of aqueous solutions of NO, and inclusion of NO-generating cells such as macrophages in cultures. All of these methods present significant limitations, such as depletion of molecular oxygen during experiments, contribution of anomalous gas-phase NO chemistry, and presence of NO concentrations that are much higher than those found *in vivo*.




To combat these problems, researchers at MIT developed a novel, controllable, membrane-diffusion gas delivery system for cell culture studies, shown in Fig. 1[3]. Designed to be inexpensive and simple to fabricate, the system is essentially a modified chemostat. It avoids gas-phase NO chemistry and is capable of producing steady-state concentrations of $O_2$ and NO that mimic those found *in vivo* at sites of chronic inflammation[4]. Using the system to study human lymphoblastoid cells (TK6), Wang *et al*. showed NO was cytotoxic only if a "dual threshold" of steady-state and total concentrations was exceeded[5], but they were unable to construct a full mechanism of NO.

**Project Origins:** As an REU student under Prof. Peter Dedon (MIT BioE), I troubleshot a copy of the system and used it to study human colon cells (HCT116), chosen because the colon is known to suffer chronic inflammation. I found that cytoxicity was largely independent of DNA lesion formation, indicating a unique pathway for each process. Thus, a full mechanism of NO action could permit the design of selective damage inhibitors the function in long-inflamed cells. I also learned of two potential problems with the system: NO could be reacting with culture medium to produce adventitious levels of reactive species (peroxynitrite, superoxide, etc.), and autoclaving the vessels could be altering the permeability of the Silastic tubing.

**Goals and Hypotheses:** My research goals are to improve the described delivery system by testing for potential problems, and to expand its use to other cell lines, with the hope of finding a full mechanistic description of cytotoxicity and DNA damage arising from NO exposure. I hypothesize that: 1. NO does not form adventitious levels of reactive oxygen and nitrogen species when delivered to cell culture medium; 2. autoclaving vessels before experiments does not significantly alter the permeability of the Silastic tubing, and; 3. cell lines from tissues susceptible to chronic inflammation will respond to NO in the same way HCT116 does, allowing for identification of biomarkers and construction of mechanistic pathway maps.

**Research Plan:** To determine if NO causes artifactual levels of reactive species in cell culture medium, I would use the system to deliver NO and $O_2$ to buffer solution (as a control) and a variety of commercial media, both with and without added serum. During delivery, I would use a protocol established by Prof. Bill Deen (MIT ChemE) to monitor the vessels with commercially available ion-specific electrodes to quantify reactive oxygen and nitrogen species (or their downstream oxidation products, in the case of transient species such as peroxynitrite).

To determine the effect of autoclaving Silastic tubing, I would use the system to deliver NO and $O_2$ to buffer solution before (as a control) and after autoclaving the vessels for various lengths of time, in each case using NO- and $O_2$-specific electrodes to measure the delivery kinetics. This would permit creation of a model of kinetic parameters as a function of autoclave duration. I am preparing for these studies with a class on biomolecular kinetics and mathematical modeling, and could further supplement this with classes on fluid dynamics if need be. Publishing this complete description of the system would have broader impacts by ensuring that it could be copied by other researchers, thereby communicating the results to a wider audience.

After these studies were complete, I would use the system to investigate the effect of NO on cell lines chosen from tissues susceptible to chronic inflammation, such as liver and lungs. I would expose wild type and p53-null cells to varying steady-state and total doses of NO, then quantify DNA damage with an ultrasensitive detection protocol developed by Prof. Dedon. This method combines radiolabeled internal standards of DNA lesions with HPLC and high mass accuracy tandem mass spectrometry to identify and quantify lesions at the femtomole level. By comparing these results to similar data for other DNA damaging agents (from Prof. Leona Samson, MIT BioE), I would first identify unique biomarkers – patterns of lesion changes that are statistically significant only in response to NO – that could provide a quantitative description of chronic inflammation in *in vivo* samples. I would then validate the biomarkers using tissue from mouse models of chronic inflammation (from Prof. David Schauer, MIT BioE).

I would then compare the range of lesions and the relation to cytotoxicity in these cell lines to HCT116, to determine if the mechanism of NO action is independent of cell type. Supplementing this study with coursework in biological network analysis (currently taking) and DNA repair, I would use this information to construct a mechanistic diagram of NO action in chronically inflamed tissues, providing both qualitative and quantitative (through use of the biomarkers) descriptions of the link between inflammation and cancer. The broader impacts are clear, as sharing this map with the research community would enhance the search for therapies.

**Anticipated Results:** My anticipated results are indicated in my hypotheses, and in each case I am prepared with a plan if those results are not observed. If accumulation of reactive oxygen and nitrogen species proved to be significant, I would investigate ways to mitigate their formation, such as the use of scavenger molecules to sequester them. If autoclaving vessels prior to experiments decreased the permeability of the Silastic tubing, I would investigate alternative sterilization procedures (such as UV light) and alternative tubing materials for the vessels. If cell lines from other tissues behaved differently from HCT116, this would indicate tissue-specific mechanisms of NO action, which would be the start of an entirely new research project.

**References:**

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**Statement of Originality:** I certify that this proposal is an independent and original work.